

GenePix Pro 3.0

MICROARRAY ACQUISITION AND ANALYSIS SOFTWARE

User's Guide

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Chapter 1

Installing GenePix Pro 3.0

If you purchased a GenePix 4000 scanner, then this chapter is redundant, as you have already installed GenePix Pro 3.0. However, if you purchased the standalone GenePix Pro 3.0 software, please use the following installation instructions.

Computer Requirements

Minimum System Requirements

- IBM-AT compatible computer with a Pentium 200 MHz or faster processor
- Windows 95 or 98 operating system (dual-boot systems are not recommended)
- 128 MB RAM
- Hard disk with 4 GB free (for image storage)
- CD-ROM drive
- 1024×768 display system with 65K colors
- Parallel port

Recommended System Requirements

- IBM-AT compatible computer with a Pentium III 500 MHz or faster processor
- Windows NT 4.0 operating system (dual-boot systems are not recommended)
- 256 MB RAM or more
- Hard disk with 10 GB free (for image storage)
- Recordable / Rewriteable CD-ROM or Jaz drive
- 1280×1024 display system with 16M colors
- Parallel or USB port

Installation of GenePix Pro 3.0

GenePix Pro 3.0 is on the included CD-ROM. Simply insert the CD-ROM into your computer and the installation program automatically starts. If the installation program does not start automatically, perhaps because you have the auto-start feature of Windows disabled, you can manually start the installation by running the Setup.exe program file on the CD-ROM. This file is found in the GenePix subdirectory on the CD-ROM.

To start the setup program manually, from the *Start* menu select “Run”, and type `x:\GenePix\setup.exe` where “x” is the drive letter of your CD-ROM drive.

When the installer has finished, you will be given the choice of restarting or shutting down your computer. Select the “Yes, I want to shut down my computer now and install the hardware” option. Once your computer has shut down, proceed to “Connecting the GenePix Scanner to

Connecting the Security Dongle

GenePix Pro 3.0 requires a security dongle to operate. If the dongle is absent you will be able to open images in GenePix Pro, but you will not be able to save your settings files or save your analysis results. In addition, you can analyze only 100 features without a dongle.

Installation of the dongle is straightforward. Simply connect it to the parallel port of your computer. If you have a printer already installed, do not worry. The dongle is a “pass-through” device and you simply connect the dongle to the parallel port, then connect your printer to the dongle. A USB-based dongle is also available. Please contact Axon Technical Support for further details if you require a USB dongle.

Starting GenePix Pro 3.0

After the successful installation of the software, you will find the entry “Axon Laboratory” in your list of Programs in the Start menu, and there will be two new icons on your desktop. There are “GenePix Pro 3.0”, and “GenePix Pro 3.0 (demo)” entries in your Axon Laboratory group, and icons for these on your desktop. Both of these shortcuts will start GenePix Pro. *The only difference between the demo version and the non-demo version is that the demo version does not interact with the scanner.*

Troubleshooting Installation

If you are having problems running the software, please consider the following:

- GenePix Pro 3.0 requires a minimum of 128 MB of RAM to acquire images. GenePix Pro is capable of analyzing data when less than 128MB of RAM is present, but the maximum image size is reduced and performance will be non-optimal.
- GenePix Pro 3.0 requires a security dongle to enable saving of results or settings files. If the dongle is not found, the software operates with limited file-saving functionality.

Contacting Axon Instruments

If you need help to resolve a problem, there are several ways to contact Axon Instruments:

World Wide Web

Home: <http://www.axon.com>.

Technical Support: http://www.axon.com/MR_Technical_Support.html.

GenePix Feedback: http://www.axon.com/GenePix_Feedback.htm.

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Chapter 2

Introduction to GenePix Pro 3.0

GenePix Pro 3.0 from Axon Instruments, Inc., is a fully featured microarray acquisition and first-pass analysis software package. Its advanced imaging and data manipulation capabilities make GenePix Pro 3.0 the software of choice for many of the leaders in microarray technology.

GenePix Pro 3.0 Features

GenePix Pro 3.0 does the following:

Imaging

- incorporates precise software control of the scan area, reducing scan time and image storage requirements;
- displays images from two wavelengths and a ratio image as they are acquired in real time;
- constructs user-configurable pseudocolor intensity and ratio images for the easy visualization of expression levels;
- includes numerous sophisticated imaging capabilities, such as color palette control, manual and automatic brightness and contrast control, and display zooming.

Analysis

- automatically constructs resizable arrays of blocks and feature-indicators for identifying and analyzing individual features on a slide;

- automatically aligns feature-indicators with features;
- automatically reads barcodes on microarray slides;
- automatically associates individual microarray features with gene names and IDs;
- allows multiple flagging of features as measures of individual feature quality;
- automatically calculates and subtracts local background effects;
- includes two instant data previewers, one for instant zoom views and primary data preview (the Feature Viewer) and one which graphs pixel intensity distributions for individual features of DNA and which includes more advanced data preview (the Feature Pixel Plot);
- is fully integrated with web-based genomics databases;
- presents the extracted data in a spreadsheet for rapid sorting;
- plots Scatter Plots of the extracted data for all the features on a microarray;
- exports intensity, ratio and quality measure data for analysis with advanced genomic information systems;
- 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;
- is backed by unlimited technical support.

Chapter 3

GenePix Pro 3.0 Software Principles

GenePix Pro 3.0 has been carefully designed so that a user can quickly acquire and analyze images of DNA microarrays without having to learn the finer details of image processing. While one can use the GenePix 4000 scanner and GenePix Pro 3.0 to acquire and analyze images without any training at all, understanding the concepts and algorithms used in GenePix Pro 3.0 is invaluable for interpreting the acquired data. This chapter introduces the fundamental principles underlying the GenePix Pro 3.0 software. In addition to this chapter, the Chapter 3 Tutorial provides information on some of the basic operations and terminology used in GenePix Pro 3.0.

Although GenePix Pro 3.0 is used to control the GenePix 4000 scanner, one can use the software purely as an analysis platform. To do this, one need only to be able to import microarray images in 16-bit TIFF format.

Spots, Features, Blocks and Arrays

Before we can discuss the finer details of image analysis in GenePix Pro 3.0, we need to become familiar with some basic terminology. This information is discussed fully in Chapter 3, so only the key concepts will be highlighted here. Within GenePix Pro 3.0 each individual spot of DNA is called a *feature*, and is assigned a circular *feature-indicator*. Feature-indicators are the primary unit of analysis in GenePix Pro 3.0. A collection of feature-indicators is called a *block*. There can be several *blocks* on a single *microarray*, depending upon the type and configuration of the microarrayer that was used to place the spots on the glass slide.

Creating and Aligning Blocks and Feature-Indicators

GenePix Pro 3.0 offers a number of methods to create and align blocks and feature indicators.

GAL File and Align Blocks

To employ the full power of automated microarray analysis possible in GenePix Pro 3.0, you can use the Array List Generator to construct a GenePix Array List (GAL) file. It is critical that the identity of the DNA in each spot is maintained throughout the analysis, so use a GAL file when you have files containing gene IDs and names for each spot on the microarray. GAL files also contain information about the printing process used on the microarray, and GenePix Pro 3.0 uses them to construct an array of blocks to match the size and positioning of printed features. Load the GAL file into GenePix Pro 3.0 with the *Load Array List* command (available under the *Open/Save* button on the right hand side of the GenePix Pro window). Once this is done, you may need to perform a very rough manual alignment of the blocks on the image before using the *Align Blocks* function. After the automatic alignment, a quick visual inspection will tell you if you need to make any further manual adjustments to feature-indicator positioning.

New Blocks and Align Blocks

Without a GAL file you can create blocks with the *New Blocks* dialog box. At a later date you can always load an array list to apply gene IDs and names to existing blocks. Align the blocks roughly on the image and use *Align Blocks* to complete the alignment. Again, after the automatic alignment you will need to inspect it for accuracy.

Manual

All the positioning and resizing of blocks and feature-indicators that GenePix Pro 3.0 does automatically can be done manually. A little manual fine-tuning of feature-indicator placing may be necessary on non-uniform arrays.

All these methods are described extensively in the Chapter 3 Tutorial.

Barcode Reading

If a microarray slide has a barcode on it, GenePix Pro 3.0 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 GenePix Pro 3.0 window, and are exported in the header of the Results file. In addition, you can use the barcode symbols as the file name of the image. To do this, check the *Barcode* option in the *Save Images* dialog box.

GenePix Pro 3.0 reads three of the most common barcode formats – code 39, code 128 and code I2of5 – which have the following properties:

Barcode Type	Numerical Characters	Alpha Characters	Special Characters	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)

Image Display

The GenePix 4000 scanner uses a simultaneous dual-laser scanning system to produce a ratio image in real time. What does the ratio image represent? This question can be answered in two ways, depending on whether your interest is technical and you want to know how the image is processed, or your interest is scientific and you want to know how the ratio represents biological information.

Technical Description of the Ratio Overlay Image

The ratio image is a standard 24-bit composite RGB (Red-Green-Blue) image. When set to the default red-green display scheme and 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. In GenePix Pro 3.0 each raw wavelength image, such as the green 532 nm image or the red 635 nm image, is collected at 16-bit resolution. However, each color channel of the RGB images is composed only of an 8-bit image.

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. To reduce each raw wavelength image to 8 bits there are two options:

- (a) compress 16 bits into 8 bits, or
- (b) selectively use only 8 of the 16 bits.

GenePix Pro 3.0 provides the ability to do either using the *Ratio Color Mapping* function.

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, GenePix Pro 3.0 can be configured to keep only the bottom, middle or top 8 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 table below:

<i>Method of Ratio Processing</i>	<i>Range of image intensity that is used</i>
Square Root Transform	0–65535
Preserve High Intensities	256–65535
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? It is important to keep in mind that 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.

This is best illustrated with an example. Suppose you have a pixel that has a green value of 15000 and a red value of 30000. If 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”, you can display all pixels from 0 to 65535. In this scenario, the pixel in question would be red, as the red channel is contributing twice as much intensity to the RGB image as the green channel. However, when you select the “Preserve Low Intensities” option the maximum pixel value is now 255. In this case, both the green and red pixels are contributing the

same intensity to the RGB image since each image is effectively treated as 255. This is because both images exceed an intensity of 255, and are thus clipped at a value of 255. The result is a ratio displayed that is yellow, corresponding to a ratio of 1 (255 / 255).

The Biology of a Ratio Image

What biological information is conveyed by a ratio image? The ratio image typically represents the level of *test* to *reference* cDNA that is hybridized to *spotted* (or arrayed) cDNA. Suppose the *reference* cDNA is labeled with Cy3 (which is excited by green laser light such as 532 nm), and suppose the *test* cDNA is prepared in the presence of Cy5 (which is excited by red laser light such as 635 nm). Both of these cDNA populations are hybridized to the *spotted* cDNA on a microscope slide. In the derived ratio image, a red spot indicates that the test cDNA for this feature is more abundant than the reference cDNA, which means that it is being expressed at a level higher than the reference; a green spot indicates that the test cDNA is being expressed at a level lower than the reference cDNA; a yellow spot means that there is no change in the expression level between the two populations of test and reference cDNA. This approach to visualizing gene expression has the great advantage of allowing one to see the dynamic patterns of gene expression.

The previous section described only how the ratio data is *displayed*. In order to measure changes in gene expression numerically, GenePix Pro 3.0 performs a number of computations on the raw data. These are described in the next section.

Analysis Algorithms

To quantify changes in gene expression accurately, it is necessary to extract the intensity of a given feature from an image, and to refine this data before a final ratio value is computed. In addition, a number of other parameters are measured to evaluate the quality of the data for a given feature. The following section describes how each computation in GenePix is performed.

Computing Feature Intensities

For each raw image, the mean, median, and standard deviation of the pixel intensities in each feature-indicator is computed. The pixels that are used to compute the standard deviation include all of the pixels that are completely inside the circular feature-indicator. Any pixel that contacts the circle that defines the feature-indicator is excluded.

Computing Background Intensities

To reduce the effect of non-specific fluorescence such as auto-fluorescence of the glass slide or non-specific binding of test and reference cDNA, GenePix Pro 3.0 uses a background subtraction method. For all analysis calculations, the background intensity is subtracted from the feature intensity before any ratio calculations are performed. The method for determining the background intensity is a local background subtraction technique. Therefore, 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.

In addition to computing the local background, GenePix Pro 3.0 reports the percentage of pixels that exceed a background-related threshold. GenePix Pro 3.0 reports 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.

Which pixels are used to compute the background? First, a circular region is drawn that is centered on the feature-indicator. This region has a diameter that is 3 times the diameter of the current feature-indicator. All of the pixels within this area are used to compute the background unless one of the following is true:

- the pixel resides in a neighboring feature-indicator;
- the pixel is not wholly outside a two pixel wide ring around a feature-indicator;
- the pixel is within the feature-indicator of interest.

In Figure 1, the black region represents the pixels used for computing the background, the dark gray region represents the pixels used for the feature

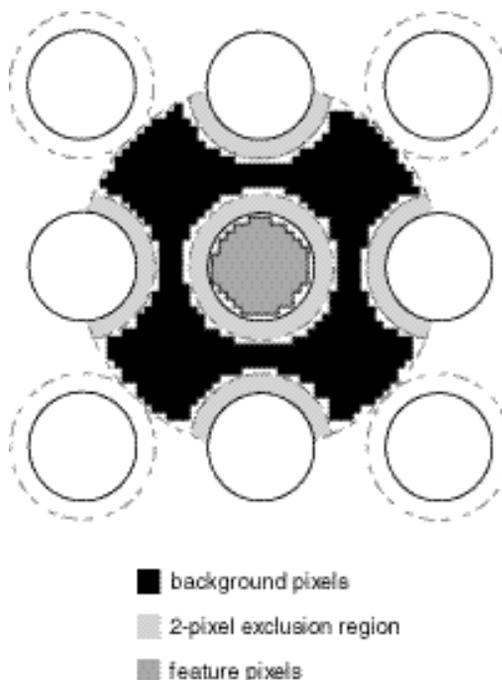


Figure 1: defining background pixels

intensities, and the light gray region represents excluded pixels.

Block Alignment and Background Thresholds

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

One important quantity used in spot-finding is the *background threshold*, which is calculated as follows:

1. the ‘mean’ of the background is estimated as the value greater than 55% of pixels (*i.e.*, 5% above the median);
2. the ‘standard deviation’ is calculated for the pixels less than the ‘mean’;
3. the *background threshold* is the ‘mean’ plus four ‘standard deviations’.

A feature is marked ‘Not Found’ if:

- no pixels are greater than the background threshold; or
- the feature diameter found is greater than the lesser of three nominal diameters (as set in Block Properties) or the diameter that would cause it to overlap an adjacent feature of nominal diameter; or
- the feature diameter goes outside the bounds set in *Options / Analysis*; or
- the feature has fewer than 6 pixels above background threshold; or
- the feature has been found in a position that would overlap an adjacent feature.

Computing Ratios

GenePix Pro 3.0 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 below, 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.

In the following discussion, I_{P,I_k} is a single raw pixel intensity at wavelength k , I_{B,I_k} is a single background pixel intensity at wavelength k , $\langle S_i \rangle_{\text{med}}$ is the median of some set of data S that contains i terms, n is the number of pixels in a feature, and m is the number of background pixels for a feature.

To get a better understanding of the ratio computations it is often useful to examine the Feature Pixel Plot (press ‘P’ when the cursor is over a selected feature-indicator). This plot shows you the actual pixel values for a selected feature, and helps to visualize how each pixel is used in each calculation.

Note: the form of all ratio calculations in GenePix Pro 3.0 is user-selectable. 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 gene expression studies one is typically most interested in the difference in expression levels between the test and the reference mRNA. This eventually translates to differences in intensity on the two images analyzed by GenePix, 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, one needs 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:

$$\frac{\langle (I_{P,I_2})_n \rangle_{\text{med}} - \langle (I_{B,I_2})_m \rangle_{\text{med}}}{\langle (I_{P,I_1})_n \rangle_{\text{med}} - \langle (I_{B,I_1})_m \rangle_{\text{med}}}$$

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 one 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:

$$\frac{\sum_{i=1}^n \left(I_{P,I_2} - \langle (I_{B,I_2})_m \rangle_{\text{med}} \right)_i}{\sum_{i=1}^n \left(I_{P,I_1} - \langle (I_{B,I_1})_m \rangle_{\text{med}} \right)_i}$$

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 when compared to calculating 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:

$$\left\langle \left(\frac{I_{P,I_2} - \langle (I_{B,I_2})_m \rangle_{\text{med}}}{I_{P,I_1} - \langle (I_{B,I_1})_m \rangle_{\text{med}}} \right)_n \right\rangle_{\text{med}}$$

Mean of Ratios

The *mean of ratios* is the arithmetic mean of the pixel-by-pixel ratios of the raw pixel intensities:

$$\frac{1}{n} \sum_{i=1}^n \left(\frac{I_{P,I_2} - \langle \langle I_{B,I_2} \rangle \rangle_{\text{med}}}{I_{P,I_1} - \langle \langle I_{B,I_1} \rangle \rangle_{\text{med}}} \right)_i$$

Regression Ratio

The regression ratio is another method of computing the ratio that does not require rigidly defining the background and foreground pixels. Therefore this is the most objective of the three methods. All of the pixels within a circle that has a radius of two diameters of the feature of interest are 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.

Because the regression ratio does not make any assumptions about which pixels are feature pixels, and which pixels are background pixels, saturating pixels can really throw the regression ratio off as they heavily weight the slope of the best-fit line. On a good array, all of the different ratio measurements 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.

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 the sum of the median or mean 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:

$$\langle\langle I_{P,I_2} \rangle\rangle_{\text{med}} + \langle\langle I_{P,I_1} \rangle\rangle_{\text{med}} - \langle\langle I_{B,I_2} \rangle\rangle_{\text{med}} - \langle\langle I_{B,I_1} \rangle\rangle_{\text{med}}$$

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:

$$\frac{1}{n} \sum_{i=1}^n \left(I_{P,I_2} - \langle\langle I_{B,I_2} \rangle\rangle_{\text{med}} \right)_i + \frac{1}{n} \sum_{i=1}^n \left(I_{P,I_1} - \langle\langle I_{B,I_1} \rangle\rangle_{\text{med}} \right)_i$$

Log Ratio

The log ratio is a base two logarithm of the ratio of medians. This type of log transformation is often used in gene expression studies as it helps to compare levels of over-expression and under-expression. For example, if you have a four-fold increase in expression, the log base 2 ratio is +2; if you have a four-fold decrease in the ratio (*i.e.* a ratio value of 0.25), the value is -2. The log base two transform is simply a quicker way to observe the similarity in magnitude between these two conditions:

$$\log_2 \left(\frac{\langle\langle I_{P,I_2} \rangle\rangle_{\text{med}}}{\langle\langle I_{P,I_1} \rangle\rangle_{\text{med}}} \right)$$

Flags

GenePix Pro 3.0 provides the ability to flag individual features manually as ‘Good’, ‘Bad’, ‘Absent’ or ‘Not Found’. The ‘Bad’ flag is often useful when doing a cursory analysis of your images and you observe a region where the feature-indicators appear sub-standard. You can quickly flag these features as ‘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 as ‘Good’. If so, a flag value of +100 is noted in the results.

‘Absent’ (numerical value -75) is applied to a feature when a GAL file is missing an entry for a feature. A feature is flagged ‘Not Found’ (numerical value -50) when *Auto Align* fails to align a feature-indicator to it.

Data Normalization

Comparing the data from different microarray experiments is a non-trivial task. Small variations in the many steps that produce a microarray image can make comparisons across microarrays problematic. Variability in the data from many microarrays can be corrected for either in the analysis stage or in the acquisition stage of an experiment.

In the analysis stage, one can improve comparisons across many microarrays by normalizing the data from each microarray. One method of normalization is based on the premise that the arithmetic mean of the ratios from every feature on a given microarray 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.

In GenePix Pro 3.0 normalization factors are calculated for each of the five calculated ratio quantities (Ratio of Medians, Ratio of Means, Median of Ratios, Mean of Ratios, Regression Ratio). The type of calculation that is performed in each case is identical; the only difference is in the source data that is used to compute the normalization factor. Note that features flagged ‘Bad’ or ‘Not Found’ are not included in the calculation of the normalization factor. If the chosen ratio for the normalization is R , then the normalization factor is:

$$1 / 10^{\frac{1}{n} \sum_{i=1}^n \log_{10} R_i}$$

where R_i is the ratio for the i th feature. The normalization factors are stored in the Results file that is saved from GenePix (which you can view by pressing the *Header* button on the Results tab), and you can choose to display a particular normalization factor by selecting it from the drop-down list on the Results tab. It is important to note that GenePix Pro 3.0 does *not* change any of the analyzed data.

Given that GenePix Pro 3.0 does not normalize the data, what is the usefulness of the normalization factor?

First, GenePix Pro 3.0 can use the normalization factor to determine if the PMT values were set correctly during acquisition. For example, if you had set the PMT voltage for the red (635 nm) channel too high, the computed ratios will be shifted to higher values. If the shift causes mean ratios greater than 2 (or less than 0.5) you are prompted to rescan your image.

While this shift can be corrected during analysis using normalization techniques, rescanning the image at the correct PMT settings ensures maximum data integrity.

Second, each normalization factor is available through the scripting interface on the Report tab, so users may wish to normalize their data in a Report.

GenePix Pro 3.0 File Formats

GenePix Pro 3.0 recognizes and uses a number of different file types. Detailed descriptions of the file formats used by GenePix can be found on our website at:

http://www.axon.com/GN_GenePix_File_Formats.html

ATF – Axon Text File Format (*.atf)

This is a tab-delimited text file format that can be read by typical spreadsheet programs such as Microsoft Excel. It is used for GenePix Array List (GAL) files, and GenePix Results (GPR) files (see below).

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 (*.gal)

The GenePix array list file conforms to the Axon Text File (ATF) format. The GenePix array list file contains entries to describe the layout of each block, and to assign names and identifiers (IDs) to each feature on a microarray.

GPR– GenePix Results File (*.gpr)

Results data are saved as GPR files, which are in Axon Text File (ATF) format (see above). The results file contains general information about image acquisition and analysis, as well as the data extracted from each individual feature.

GPL – GenePix Lab Book File (*.gpl)

The GenePix Lab Book is a binary file that contains a fixed-size structure for each line in the Lab Book.

GPS – GenePix Settings File (*.gps)

GenePix acquisition, analysis and display settings are saved as binary GenePix Settings Files. Settings are organized into a number of 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 wish to open.

Acquisition settings include which laser was enabled during the acquisition, the PMT voltages, 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 color mapping.

JPEG – Joint Photographic Experts Group (*.jpg)

Images can be saved in the JPEG format, which is a lossy compressed image file format. GenePix implements minimal JPEG compression, which is enough to reduce image file size significantly, but which removes only a small amount of data from the image. However, we recommend that you do not use the JPEG format to archive images that are to be analyzed later. Rather, use the JPEG format to store images that are to be used in presentations.

TIFF – Tagged Image File Format (*.tif)

Images acquired in GenePix 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 GenePix 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 wish 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.

GenePix 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.

Chapter 4

GenePix Pro 3.0 Tutorial

About This Tutorial

GenePix Pro 3.0 has been carefully designed to maximize your efficiency and to minimize the time you spend doing routine microarray-related tasks. This tutorial is designed to get you up and running as soon as possible by walking you through a real experiment with GenePix. It will show you the steps involved in loading your first microarray and seeing your first microarray image. We will also guide you through the steps involved in doing a microarray analysis, and saving your data for further analysis. For this reason, we have kept the detail in the tutorial to a minimum.

The tutorial is organized into three main sections. The first section ‘Image Acquisition’ takes you from starting up the GenePix scanner through to scanning a microarray and saving your images. If you are using GenePix Pro as a standalone application, you may wish to skip this part. The second section describes image analysis using GenePix. The third section describes a number of special features in GenePix that you may use regularly.

At various points you may feel that you need more information before continuing with the tutorial procedures. This Guide and the on-line Help each contain a glossary of important terms that you might find useful while working through this tutorial.

If you require more information about details of the software interface, consult the on-line Help which you can call up at any time from within GenePix Pro 3.0 by pressing “F1”.



If you would like more information about the technical details behind the GenePix microarray scanning system, consult Chapter 2 in this User's Guide, or Chapter 4 in the GenePix 4000

1. Image Acquisition

Starting GenePix 4000

The GenePix 4000 scanner must be turned on before your computer is powered up so that the computer can identify that the scanner is connected and operational. If you have not yet performed the GenePix 4000 installation, you may wish to refer to the GenePix 4000 User's Guide. Once Windows has successfully started you can start GenePix Pro 3.0, either through the icon on the desktop or the icon in the *Start Menu / Program Files / Axon Laboratory / GenePix Pro 3.0* sub-menu.

GenePix Demo Mode

During the installation of GenePix two icons are created, one of which is labeled "Demo". The demo version provides exactly the same functionality as the full version, except that it does not interact with the GenePix 4000 scanner. Like the non-demo version, the demo version requires a dongle for full functionality.

You may want to use the Demo version in a number of circumstances.

If you are acquiring image data with one computer and then performing analyses on another, running the non-demo version on the second machine would result in prompts about the hardware not being attached. Simply select the *Don't look for scanner at startup* option to permanently eliminate these prompts and retain full analysis functionality. Running the demo version, you have full analysis functionality without having to worry about hardware.

Second, if you are acquiring and analyzing on the same computer, there is an advantage in using the Demo version only for analyses, particularly if they are lengthy. While the full version of GenePix Pro 3.0 is running, the scanner's lasers are fully powered (although the lasers power down if the scanner is left idle for two hours). Exiting from the full GenePix Pro 3.0 software powers down the lasers on the scanner immediately. In the long term, switching to the Demo version for analyses may significantly extend the lasers' operating lifetime.

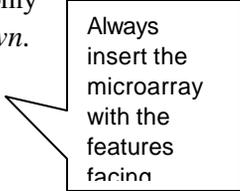
The GenePix Pro 3.0 Interface

One of the features of GenePix Pro 3.0 that you will notice immediately is that it does not have any of the usual menus used by Windows programs. Instead, all of the commands necessary to operate the GenePix hardware and to perform a precise analysis are immediately available via colorful and intuitive buttons. Furthermore, the various GenePix functions are organized using tabs, instead of separate windows. Initially, most of your time will be spent acquiring and analyzing data in the Image tab.

For users who are more comfortable using the keyboard than the mouse, there are keyboard shortcuts for all GenePix Pro 3.0 functions. Many of these shortcuts are in the popup ToolTips that appear when you rest the mouse cursor over a toolbutton. There are even several special shortcut keys for power users that are designed to be real time-savers. These shortcut keys are mentioned throughout the tutorial and are summarized in the on-line Help.

Preparing Your Microarray Slide For Scanning

GenePix is designed to accommodate $1 \square \times 3 \square$, $25 \text{ mm} \times 75 \text{ mm}$, and $26 \text{ mm} \times 75 \text{ mm}$ glass microarrays. Inserting and removing a microarray slide is straightforward. The only rule to remember is *always insert the microarray with the features facing down*. The optics of GenePix are designed such that laser light is directed up from the bottom of the GenePix 4000 onto your microarray, and therefore the features must be facing down. The orientation of your microarray is critical for the successful operation of GenePix.



Always
insert the
microarray
with the
features
facing
down

Inserting the Microarray

Before inserting and removing a microarray, GenePix Pro 3.0 must be running. To insert the microarray, simply slide open the scanner door, and the slide holder moves automatically to the loading position. When the slide holder has stopped moving the yellow eject light blinks, indicating that it is safe to load the microarray. Open the sliding GenePix door and lift the locking latch to unlock the door of the slide holder. Whenever the sliding GenePix door is open, the laser light is automatically blocked, so there is never any danger to the user of being exposed to laser light.

Now that the door is open you can easily insert the microarray into the holder. The microarray is seated on three sapphire micro-spheres to ensure its precise alignment. Place the microarray on the micro-spheres *with the features facing down*. Once the

microarray is seated, you can close the door and depress the locking latch until it locks (you will hear an audible click) to indicate that the slide has been successfully inserted into the GenePix scanner. After closing the sliding GenePix door, GenePix is ready to scan your microarray.

Removing the Microarray



Eject

Removing the slide is simply the reverse of inserting it. To eject the slide you can either open the sliding GenePix door, or press the *Eject* button located on the right side of the GenePix Pro 3.0 window. After the *Eject* light has illuminated you can open the sliding GenePix door, lift the latch, and raise the slide holder door to remove the slide.

Using GenePix to Acquire an Image

When a microarray has been successfully inserted into GenePix, the next step is to acquire an image. While acquiring an image is very straightforward, there are a few considerations that should be made prior to starting an acquisition.

Setting the Scan Mode and Scanning Region

Preview
Scan

GenePix can scan a microarray in one of two modes, and can scan either an entire microarray or just a portion of a microarray. Typically you will first select the low resolution *Preview Scan* button to acquire quickly a rough representation (40 μm per pixel) of the layout of the microarray. After completing a preview scan, you will re-scan either the entire microarray or a portion of the microarray at high resolution (10 μm per pixel). If you want to re-scan only a portion of the array you can specify the region to scan using the *View Scan Area* button. In this mode you can draw a region to be scanned; if you do not specify a region, an image of the whole slide is acquired. After specifying the scan region, pressing the *Data Scan* button begins a scan over just the portion of the microarray that is delineated by the scan region.

View
Scan AreaData
Scan

During scanning, all of the image display controls and zooming functions are fully operational. A thorough discussion of these items is found in the “Image Analysis” section below. If you have *Ratio* selected in the *Image* group at the top left of the Image tab, during scanning you can see the ratio image being built up in real time. You can also quickly flip between the display of each of the individual wavelength images, and the preview image, if you acquired one.

Optimizing Hardware Settings

The *Hardware Settings* dialog box controls the amplification (gain) of the photomultiplier tubes (PMTs), which is adjusted to maximize the dynamic range of the scanner. To change the amplification, increase or decrease the voltage of the PMT by typing in a new voltage, or by using the spinners to increase or decrease the current voltage. Set each PMT such that there are only a few pixels that are saturating in each image. Saturating pixels represent a condition whereby 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. These pixels are visible as white pixels in the image; alternatively, you can determine the number of saturated pixels from the histogram.



Hardware
Settings

The histogram is found in the Histogram tab. It plots the distribution of all of the pixels from each wavelength for the currently displayed images. Saturated pixels appear on the far right of the histogram, as saturation corresponds to an intensity value of 65535. It is important to realize that the histogram plots only the data for the currently displayed image. If you have zoomed in on an image, the histogram represents only the region that is currently visible. The histogram is a useful tool to see how much of the dynamic range is being used. In addition, the histogram shows how much overlap you have in the red and green channels. This is an indication of color balancing. For example, the ideal scan is one whereby the peak of the green histogram overlaps quite closely with the peak of the red histogram. This indicates that the two channels are quite similar in intensity. In reality, often one channel is slightly shifted relative to the other due to it having higher background fluorescence. In practice, you may perform a preview scan and optimize the PMT settings during this scan using both the histogram and the displayed image.

In addition to controlling the voltage of the PMTs in *Hardware Settings*, you can also turn each laser on or off independently. To maximize your signal-to-noise you may want to average multiple lines. Keep in mind that as you increase the amount of line averaging you will correspondingly increase the amount of time needed to complete each scan.

You can save your acquisition settings for future use by selecting the *Open/Save* button, and choosing *Save Settings* from the popup menu. This will save your hardware settings, as well as analysis and display parameters, in



Open/Save

a GenePix Settings (GPS) file. These settings can be opened later by clicking the same *Open/Save* button, and choosing *Open Settings*.

Saving an Image



Once your microarray has been scanned, the next step is to save the image. Pressing the *Open/Save* button and select *Save Images*, which brings up a dialog box that offers a number of flexible options to control how your data is saved.

Choosing A File Format

By default, all images created in GenePix are saved as 16-bit multi-image TIFF files, which we recommend using. Each single multi-image TIFF file contains the images for each individual wavelength scan. The ratio image is not saved, but is recomputed when the multi-image TIFF file is opened in GenePix.

GenePix also provides the option to save each image component of the acquired image as a separate file. There are a number of reasons why you may wish to do this. Some graphics applications cannot read multi-image TIFF files; if you use one of these, you will have to save the images separately if you are going to import them into such a program. Second, you may want to save just the ratio image for presentation purposes. You can only save the ratio as a single-image file; it cannot be saved in the multi-image TIFF file. However, it is important to realize that *if you do not save the raw wavelength images, future analysis of these images will be impossible*. Therefore we recommend using the multi-image TIFF format for storing your data, and using the separate individual file format for creating presentation figures.

Do NOT
use the
JPEG
format for
archiving
data!

In addition to saving your data in TIFF files, GenePix also lets you save the image in JPEG format. This file format is an efficient but *lossy* compression format: the final file size is significantly reduced, but the compression process marginally affects the raw data. 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.

Setting File Names

GenePix provides you with three additional options to control file names. You can check the *Date Prefix* option to prefix the file name with the current date (YYYY-MM-DD). Checking the *Numeric Suffix* option adds a four-digit suffix to the file name, beginning at 0000. Checking the *Barcode Prefix* option prefixes the file name with any automatically scanned barcode symbols from your image. GenePix 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, GenePix adds the wavelength of the image as a suffix to the file name.

2. Image Analysis

Once an image has been acquired, the next step is to view the image and measure the signal intensity of each feature. GenePix Pro 3.0 has a number of functions to perform such tasks efficiently. For ease of discussion, we will work through the analysis of one of the demonstration images supplied with GenePix. If you are feeling slightly more adventurous, you can follow along using the image that you just acquired.

Opening an Image

If you have just acquired an image, the screen will already display an image that you can work with. If not, press the *Open/Save* button to open an image that has been previously saved to disk. For example, open one of the sample files found in the “Axon\Data” directory.



Controlling the View of the Data

Image Display and Color Control

Now that an image is displayed, there are several options available to modify the view. You can easily switch between the different images by selecting the different radio buttons in the *Image* group at the top left corner of the GenePix Pro 3.0 window.

GenePix lets you control the display of each image independently. These image display controls are also available in real time during an acquisition. It is worth experimenting a little with the various display settings to find those with which you are most comfortable. For example, green–blue with inverted color is good for distinguishing features from background at high magnifications.

You can invert the colors, similar to a negative for a photograph, using the *Invert Color* button.

If you are viewing a raw wavelength image, you can select a monochrome, grayscale or rainbow view using the *Color / Mono* button.

Color blind?
Use a different color mode!

If you are viewing a ratio image, you can specify the type of overlay scheme to use with *Ratio Color Mapping*. By default a red–green overlay is used, which results in an image composed of red, green, and yellow pixels. Alternatively, you can select a green–blue or red–blue overlay. The alternate types of overlay may be particularly useful for people with some forms of color blindness.

Standard brightness and contrast controls are also available. They can be set manually using the sliders marked  and , or automatically optimized for a single image by pressing the *Autoscale Display Settings*  button. To reset to the default values press the *Reset Display*  button.

White pixels reveal hardware saturation

While the raw intensity images are shown as shades of the colors selected for the color mapping, you may occasionally see white pixels. White pixels represent saturation, which indicates that their pixel intensity exceeds the range of the optical detectors or the analog to digital converters. In such cases it may be necessary to reduce the settings for the photomultiplier tubes (PMTs) to ensure that a valid ratio can be computed. This was discussed above in the description of the *Hardware Settings* dialog box.

What do the different images represent?

There are four images in GenePix. The Preview Image is a ratio image derived from a 40 μm resolution scan. It is a quickly scanned image that you can use to preview a slide and set the Scan Area for a Data Scan. The two single-wavelength images (typically 635 nm and 532 nm) are obtained from 10 μm resolution scans; they are 16-bit grayscale images that are displayed in one of four ways using the *Color / Mono* setting. Ratio images are created by overlaying the single-wavelength images onto each other to form a 24-bit RGB image. However, the ratio data are obtained by computationally dividing the two 16-bit images to produce ratio values. The ratio colors (set with the *Ratio Color Mapping* command) represent different levels of relative gene expression. See Chapter 3 for more information.

Zooming the Display and Using the Feature Viewer



Zoom
Mode



Zoom
Full Scale



Undo
Zoom

To increase the magnification of the image being displayed, you can select *Zoom Mode* and then draw a box around the area that you would like to view at higher magnification. After zooming an area you can return to the previous magnification using the *Zoom Undo* button. Alternatively, you can reset the display to its default magnification using the *Full Scale* button. You can quickly change the mouse cursor to zoom mode at any time by pressing “Z”, or by holding down the “Alt” key.

In addition to zooming the display manually, a *Feature Viewer* window is constantly displayed at the bottom left of the Image tab. The Feature Viewer employs a user-defined zoom factor (set in the *Options* dialog box, on the *Customize* tab) to display a zoomed view of the image that is currently under the mouse cursor. It also lists important information about each feature, such as the (x, y) coordinates of the cursor, and various ratio and intensity values. Once an analysis array has been created (see the next section), when the mouse is placed over a feature-indicator the Feature Viewer reports the intensity of the pixels contained within the feature-indicator, its associated background intensity level, the precise location of the feature-indicator in the analysis array, and gene IDs or names that have been defined for each feature.

Use the
Feature Viewer
to
visualize a
feature
quicklv.

Creating and Using an Analysis Array

In order to measure the intensity of individual features you must first create an analysis array, which is used to define the dimensions and location of each feature. A detailed discussion of the algorithms used by the array can be found in Chapter 3. For the purposes of this tutorial, we simply need to know the terminology that is used to define an analysis array, and the tools that are used to create and manipulate the array.

Spots, Features, Blocks and Arrays

In GenePix Pro each individual spot of DNA (a *feature*) that is deposited on a microarray glass slide is assigned a *feature-indicator*. Feature-indicators are the primary units of analysis in GenePix Pro. A collection of feature-indicators is called a *block*. There can be several blocks on a single microarray, depending upon the type and configuration of the microarrayer that was used to place the spots on the glass slide. Shown right is a small hypothetical 400-feature array, composed of 4 blocks, each with 100 features.

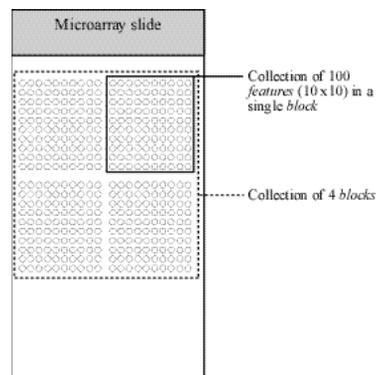


Figure 2: hypothetical array of blocks

Creating Blocks and Feature-indicators

To create a block of feature-indicators you can use one of two methods. You can generate the blocks with the *New Blocks* command, or you can automatically create the blocks using *Load Array List* (available via the *Open/Save* button) to import a GenePix Array List (GAL) file. In either case, once a Block is created it can be manually resized, rotated and aligned with feature-indicators using simple mouse and keyboard controls, and its properties can be edited by simply double-clicking on it.



New
Blocks

Example 1: Manual Block Creation

To create the blocks manually press the *New Blocks* button. Enter the number of blocks that you would like to use by specifying the number of rows and columns of the array of blocks. To determine the position of the blocks, specify the distance between them (see Figure 3). If you do not have these distances from your array manufacturer or spotter, you can measure these distances

directly from the microarray image using the coordinates in the Feature Viewer.

Once you have outlined the layout of the blocks, you then specify the layout of the individual feature-indicators in each block (see Figure 3). In addition to setting the various parameters that determine the number and location of feature-indicators, you also specify the diameter of the circular feature-indicator, and any rotation of the block relative to the horizontal.

Don't worry too much about initially getting the spacing precisely correct. You can quickly adjust the spacing by stretching the block corners with the mouse, or by using the equivalent

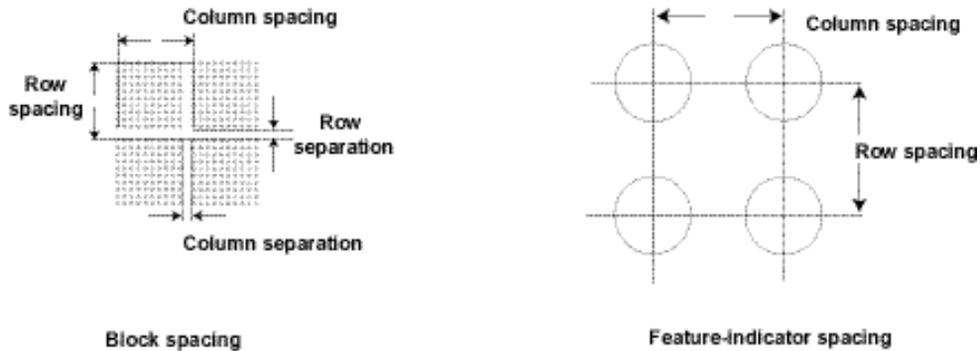


Figure 3: block and feature-indicator spacing

keyboard shortcuts.

GenePix allows three different arrangements of feature-indicators within blocks: the common rectangular layout, and two different “orange packing” layouts where the feature-indicators are placed on a hexagonal array. In the orange packing layouts, columns are vertical, but rows are zigzag (see Figure 4). Use the different arrangements depending on how your microarray slide was spotted.

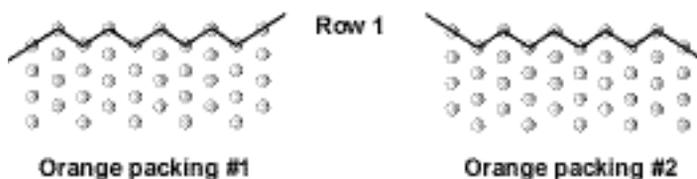


Figure 4: orange-packing layouts of feature-indicators

Example 2: Manual Block Creation in Replicate Mode

GenePix has a special mode that can be used for easy block creation. First, create a single block using the *New Blocks* dialog box as described above. Select Replicate Mode  from the mode buttons. Click anywhere on the Image Tab, and a Block is created with its top left feature-indicator at that point.

Example 3: Automatic Block Creation

As a powerful alternative to creating blocks manually, GenePix can automatically generate blocks using information that is contained in a GenePix Array List (GAL) file. GAL files are used to link the information from the microarraying process to the analysis. They speed the analysis process and add a great deal to the data that are extracted from the microarray. For example, when a GAL file is imported, the Feature Viewer reports the gene ID or name associated with a feature when the mouse is placed over it, and an Analysis operation will export these names to the Results tab together with all other extracted data.

The format of the text file is very straightforward and it is possible to prepare such a file in a standard spreadsheet program. The format of GAL files is described on the GenePix File Formats web site. GenePix also includes an

Array List Generator that takes well-plate files with gene IDs, names, and printing information and creates GAL files. It is very similar to the *New Blocks* dialog box, but includes the ability to import well-plate files.

Select the *Load Array List* command (available under *Open/Save*) and open the demo GAL file (demo.gal). This procedure automatically creates blocks, and assigns gene names and identifiers (if they are in the GAL file) to each spot. If you already have the same number of blocks and features on the image (say, from example 1) you are offered the option of replacing them, or of applying gene names and identifiers from the GAL file to the existing blocks. If particular features are missing in the GAL file, they are flagged as *absent* when the blocks are created: these feature-indicators are drawn with a dashed circle.

Many powerful GenePix functions depend on an array list being loaded. In particular *Find* and *Go To Web* require gene names or IDs.



Regardless of how the blocks are created, it is possible that they will obscure the image. Therefore, you can toggle the display of the analysis array on and off by pressing the *View Blocks* button.

Creating a GAL File with the Array List Generator



The Array List Generator converts plain text files or ATF files into GenePix Array List (GAL) files. Although you can create GAL files manually in a spreadsheet program such as Microsoft Excel, 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 GenePix Pro is installed, a number of sample 384-well plate and 96-well plate files are copied to your **.../Axon/Params** directory so that you can learn how to use the Array List Generator and Well Converter. You may wish to open one of the '384 Plate...' text files with a text editor such as NotePad to see the file format for yourself: it simply 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, click the *Add* button to open a File Open dialog box. Select all the '384 Plate...' text files by holding down the Shift key and clicking the first file, and then the last file. Once all the files are selected, click the *Open* button. All the files are added to the File list. You can now edit the configuration of the Blocks by changing the Block Properties on the right-hand side of the Array List Generator window. The Block configuration is Previewed at the bottom of the Array List Generator window. Once you are satisfied with the configuration, click the Create Array List button and save the file in a location and under a file name of your choosing.

Use the *Web Address* dialog box to set a default URL to be used with the GAL file. With the GAL file loaded in GenePix Pro, the URL is used by default whenever you look up a gene name or ID on a web genomics database with the *Go To Web* command.

If you have 96-well plate files, use the *Well Converter* to convert them into 384-well plate files. Open the *Well Converter* from its button in the Array List Generator. *Well Converter* is explained fully in the on-line Help.

Working with Blocks

Now that you have defined some blocks, they need to be aligned precisely with the features on the image. To operate on blocks you need to be in *Block* mode (Hot Key "B").

Zooming, Moving, Resizing and Rotating Blocks

To select multiple blocks simply hold the Shift key while left-clicking on a block. You can move a single block by dragging; you can quickly copy a block by holding the Ctrl key and then dragging and dropping a selected block. Resizing a block is also a simple procedure: grab an edge of a block with the mouse, and drag it until it is resized. To deform a block, hold down the Ctrl key, grab a corner of a block with the mouse, and drag it. To rotate a block with the cursor, grab a Rotate vertex  and drag through an angle.

Keyboard Shortcuts

GenePix employs a large number of keyboard shortcuts to speed up the process of aligning blocks with features on an image.

Quick Zooms

While you can use the *Zoom Area* tool for zooming, GenePix has some special zooming functions that are designed specifically for adjusting block locations. To use these special functions, first select a single block. With a single block selected the following Hot Keys are active:

- “1”: zoom top left quadrant of the selected block;
- “2”: zoom top right corner;
- “3”: zoom bottom left corner;
- “4”: zoom bottom right corner;
- “5”: zoom and center whole block;
- “>” or “<” : next or previous block.

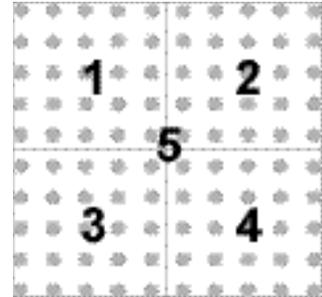


Figure 5: zooming Hot Keys

Use the Hot Keys to zoom in quickly on an area.

Once you have zoomed the block so that you can see the location of feature-indicators and how well they align with features in the image, you can move the block either by dragging it using the mouse, or by simply pressing the arrow keys on the keyboard.

Moving and Resizing

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:

Move: arrow keys

Resize: Ctrl+arrow keys

Rotating Blocks

With a block selected, you can rotate it 0.1 degrees at a time with the “+” and

Fine-tuning Block Placement

A typical procedure to align a single block would be as follows. First, align and size a block roughly with the mouse. Next, use the “1” Hot Key to focus on the top left

quadrant. Since a block is anchored at the top left corner, align the top left-most feature-indicator with the top left-most feature by using the arrow keys. Jump to the top right quadrant by pressing the “2” Hot Key. Resize the block vertically with Ctrl+up and down arrow keys; resize the block horizontally with Ctrl+left and right arrows; rotate the Block into its correct position with the “+” and “-” keys. You can then jump to the remaining two corners (“3” and “4”) to finalize the positioning and sizing of the block. Press “5” to get a lower magnification overview of your block placement. You can then jump to the previous or next block using the “<” and “>” arrow keys.

If you have multiple blocks, it is easy to setup the size and rotation of a single block and then apply these settings to all of the blocks. Simply double click the block that has been optimized. This will open the *Block Properties* dialog box. You can then select the “Apply to all” option, and the settings for the block will be propagated to all of the other blocks.

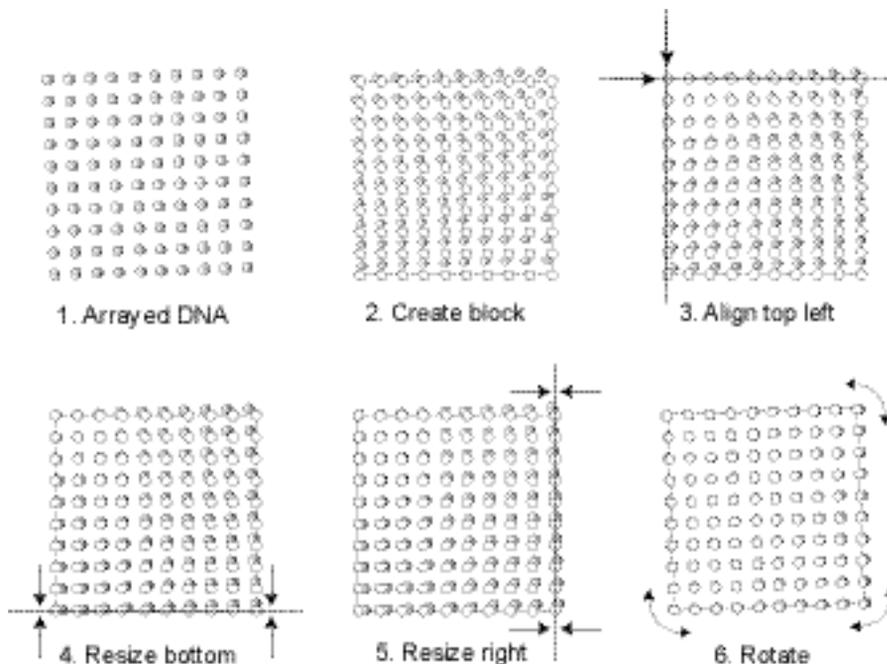


Figure 6: block-manipulation procedure



Align Blocks

Clicking the *Align Blocks* button brings up a number of alignment options, divided into two categories. The options in the first group perform two independent alignments, while the options in the second group perform only one. GenePix Pro uses two alignment algorithms: one for finding the blocks of feature-indicators on an image, and one for aligning the feature-indicators within the blocks with features on the image.

Find Blocks places each Block in its optimal starting position for the alignment of features. It does not move feature-indicators within Blocks, nor does it rotate Blocks. If the Blocks of features in your image are severely rotated, you may have to rotate the Blocks manually before attempting *Find Blocks*. Place each Block roughly over a Block of feature indicators. The better the starting position, the better the alignment.

Align Features works best when the initial position of a block satisfies the following two criteria:

- each feature-indicator partially overlaps its associated feature (say, about half a feature-indicator diameter overlap);
- the feature-indicator diameter is slightly smaller than the average feature diameter.

If your arraying process has produced features that vary greatly in size, you can change the maximum and minimum diameter settings in the *Options / Analysis* dialog box. By default, GenePix Pro varies diameters by a factor of two in either direction. You can disable diameter resizing altogether during *Auto Aligns* by unchecking the *Resize features* box.

Working with Feature-indicators

Manually Moving, Resizing, and Fine-tuning Feature-indicators

After an *Align Blocks*, you may need to fine-tune the alignment manually. For example, it may be necessary to align manually features that are flagged *Not Found* by *Align Blocks*. This is easily accomplished using the *Feature* mode.

Forgot the Hot Key? Use the on-line Help or a right mouse

After selecting *Feature mode* click on one, or many, feature-indicators that you would like to move or resize, and they are highlighted. Use the arrow keys to move or resize them (these keys are also listed in the right mouse menu in Feature mode):

Move: arrow keys

Resize: Ctrl+arrow keys

You can also use the mouse to manipulate feature-indicators. You can drag a feature-indicator to move it, and resize it by holding down the Ctrl-key and spinning the mouse-wheel. (Note that the mouse wheel is found on a standard Microsoft Intellimouse, and other compatible mice). In GenePix Pro 3.0 the resizing is the same for both *x* and *y* dimensions, *i.e.* feature-indicators are always circular. The diameters of feature-indicators are reported in the Results spreadsheet. Feature-indicators should fit closely around features.

Flagging Feature-indicators

GenePix provides the ability to “flag” individual feature-indicators. For example, you may find some features should be ignored during your final analysis because of imperfections on the microarray. You can mark individual feature-indicators as “good” or “bad” features by using the right mouse menu when in Feature mode on the Image tab. After selecting a single feature-indicator or a group of feature-indicators, select the “Flag Bad” option in the right mouse menu. Alternatively you may want to highlight certain positive feature-indicators, and you can easily do so using the “Flag Good” feature tool.

If *Align Blocks* fails to find a feature, the feature is flagged “Not Found”. When a GAL file is imported and features missing from the block descriptions, they are flagged “Absent”. Both of these flags can also be applied manually in Feature mode: select *Flag Absent* or *Flag Not Found* from the right mouse menu.

All flags are stored in the final analysis with numerical equivalents: “Good” is given the value 100, “Bad” is given the value -100, “Not Found” is -50, “Not Found” is -75, otherwise is 0.

Resetting Flags and Positions, and Undoing Previous Actions

To reset the flags or nudges for the feature-indicators on selected blocks, open the *Block Properties* dialog box (from the right mouse menu). The *Clear Flags* and *Clear Nudges* buttons clear the blocks and close the dialog box.

If you do not want to reset an entire block, you can reset just your previous action using the *Undo*  command. *Undo* tracks all of the changes that are made to blocks in Block mode, and provides an easy way to revert to a previous setting. The *Undo* command is separate from the *Undo Zoom* command; the latter only tracks changes in the magnification of the image display.

Undo
operate
s in
Block
and
Feature

Saving the Positions of Blocks and Feature-indicators

After you have finished aligning your analysis array, it is important to save its configuration using the *Save Settings* command, accessible under the *Open/Save* button. 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 special color adjustments that you have made.



When you reload a settings file you are given the option to load only those portions of the settings file that you would like to use. By default all options are enabled, and this is likely to be the most common use. However, you may have created a settings file that works well for a given microarray, but you do not want to use the red-blue color palette that your color-blind colleague prefers. In this case you would simply uncheck the *Display* option in the *Open Settings* dialog box before you open the settings file.

Settings
files store
acquisition
, analysis
and
display

Performing the Analysis and Viewing the Results

After aligning the analysis array, the analysis is performed by pressing the *Analyze* button. The analysis is performed on the raw images in each feature-indicator, and the results and subsequent computations are sent to the Results tab spreadsheet. After the Analysis a temporary JPEG file of the area of the image analyzed is saved for use in the Feature Viewers in the Results and Scatter Plot tabs. The image is saved with the brightness, contrast and color settings at the time of the



Analysis. It can be saved permanently with the Results by checking the *Save a JPEG image* option in the *Save As* dialog box on the Results tab.

In addition to the basic raw data and derived ratio measurements, the Results spreadsheet also shows the location of each feature-indicator, textual information from the Array List file (such as gene names and ID), and any flags from the array.

You can quickly sort the data in the spreadsheet by clicking on a column title. Clicking the same column title again will sort the data in the reverse order. If you wish to group a number of discontinuous rows together, select them by holding down the Ctrl key, and then press the *Group Data* button. You can reorder the columns by simply dragging and dropping them directly in the Results spreadsheet, or by using the *Choose Columns* dialog box. In *Choose Columns*, reorder columns by clicking the up and down arrows, and show and hide columns by moving them between the *Available Columns* and *Visible Columns* fields with the horizontal arrows.

Use the *Filter Data* dialog box to selectively display particular features, depending on how they are flagged. While Filter Data does not change the underlying data set or the Results file that is saved, it can be extremely useful for displaying just a subset of data in which you are interested.

Click on a record in the Results spreadsheet to view its associated feature in the Feature Viewer. The same feature is highlighted if you switch to the Scatter Plot tab.

Just as you can flag features in the Image tab, you can also flag them after an analysis in the Results tab and the Scatter Plot tab. For example, in the Results tab you may wish to sort on a ratio value, and then flag all those features ‘good’ that fall above a certain value. Hold down the Shift key to select many features together, and then select ‘Flag Good’ from the right mouse menu. Similarly, in the Scatter Plot tab you can highlight a group of features by holding down the mouse button and dragging it around the features, and then select ‘Flag Good’ from the right mouse menu.

Web Interactivity

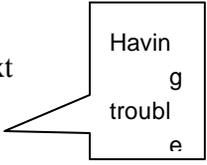
GenePix Pro 3.0 is closely integrated with the web, adding another dimension to the analysis of your microarray data. If you have a GAL file loaded (such as the demo GAL file) that contains gene names and IDs, you can select a feature, open a web genomics database and automatically look up the gene. This is all done with the *Go To*

Web command, accessible from the right mouse menu in Feature mode on the Image tab, and also available from the Results and Scatter Plot tabs.

The target web site is set on the *Options/Analysis* tab. By default, GenePix Pro has a number of web sites entered in the list. You can select one of these databases, or you can enter your own URL. After selecting *Go To Web*, GenePix Pro launches your default web browser and goes to the selected URL. Simply select a feature in the Image tab, Results tab or Scatter Plot tab, and select *Go To Web*.

Saving and Exporting the Data

Once the analysis has been completed, you can save the data in a tab-delimited text file using the *Save As* button in the Results tab. You can also copy the results of a GenePix analysis by copying the data to the clipboard from which it can be pasted into another application such as Microsoft Excel. You can either copy the entire spreadsheet or a number of rows. Simply select the rows to copy prior to pressing the *Copy* button (or its Hot Key “Ctrl+C”) on the right-hand side of the GenePix Pro window. By default all of the data are copied. However, if you have selected a single row or group or rows and then decide that you want to copy all of the data, you can select all of the data by pressing “Ctrl+A”.



Having
trouble

If the buttons for exporting the data are disabled, this is because you currently do not have a GenePix dongle attached to your computer. The dongle is a software piracy protection device. GenePix will not allow you to export data if a dongle is not attached to the parallel port.

3. Special Functions

The following is a description of other features of GenePix that you may use on a regular basis.

Feature Pixel Plot

The *Feature Pixel Plot* is a powerful graphical tool to help you evaluate the validity of the placement of your feature-indicators. You can access the Feature Pixel Plot in the Image tab by double-clicking on a single feature when in Feature mode, or by pressing “P” when the cursor is over top of a feature in any mode (an array of blocks must be defined on the image). The Feature Pixel Plot graphs the distribution of the pixels within the selected feature-indicator. For example, it



The Pixel Plot
helps to
validate your
data.

is easy to visualize the population of pixels that are contributing to the feature intensity and the population of pixels that are contributing to the background. The Feature Pixel Plot also shows derived ratio measurements, and helps you to visualize what these computations are based on. The derivation of all ratio measurements is discussed in further detail in Chapter 3.

Histogram

The Histogram display is a powerful tool that graphically reveals the distribution of pixels for each raw wavelength image. The display conveniently color-codes each distribution according to the *Ratio Color Mapping* used to create the ratio image. The display also has intuitive zooming controls to allow you to focus quickly on an area of interest, such as saturation and baseline.

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, fewer pixels are used to construct the Histogram.

You can use the histogram to see how many pixels are saturating. Zoom in on the far right edge of the histogram, where the saturating intensity of 65535 is located, to see the proportion of saturated pixels.

The histogram is also useful to check the balance of the PMT settings for each of the lasers. For example, if the peak of the red histogram is significantly shifted to the right of the peak of the green histogram, this suggests that you may need to increase the PMT settings for the green channel (see above, “Optimizing Hardware Settings”).

Scatter Plot

Unlike the Feature Pixel Plot, which plots the pixel intensities from a single feature of DNA, on the Scatter Plot tab you can simultaneously plot a quantity from every spot of DNA on a microarray. You can plot any extracted analysis quantity against any other quantity: mean intensities, median intensities, ratios of means, ratios of medians. Clicking on a point on the Scatter Plot also displays the associated feature in a Feature Viewer. The Scatter Plot is a powerful data display feature: when plotting median intensities from the two wavelengths against each other, for example, the diagonal through the origin separates over-expressing from under-expressing features.

The Scatter Plot and the Results spreadsheet are fully integrated: select a number of features in one tab and when you switch to the other tab they are highlighted.

Workflow Options

GenePix Pro 3.0 includes a number of options designed to automate routine tasks. The most obvious of these is the *Preview Scan then Data Scan* button. 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.



Other Workflow options can be set on the Workflow tab in the *Options* dialog box. These options allow you to save images automatically after they are scanned, to eject slides automatically after scans, to align Blocks automatically after a data scan, to analyze data automatically after a scan or alignment, and to save Results automatically. To Auto Align and Analyze automatically after a Data Scan using Workflow options, be sure to load a GAL file before the scan. You will also have to check the third and fourth Workflow options: ‘Align features after a data scan’ and ‘Analyze after a data scan’.

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

Report Tab and Scripting

The Report tab allows full scripting of all GenePix Pro acquisition and analysis functions. It 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. To demonstrate some of the functionality and power of the GenePix Pro Report tab, a number of sample scripts are installed with the program. These are always available from the Report tab by clicking the *Home* button.

With a Results file open in the Results tab, click on the ‘Example Results’ hyperlink in the Report tab. This opens a sample Report that constructs a table of Results data. Click the *Home* button on the left of the Report tab to return to the *Default Installed Scripts* page.

The Example Scripts demonstrate how to use some properties and methods of the GenePix Pro Object Model. The Advanced Scripts are included to suggest various sorts of real Reports that you may wish to write, and to provide models of more complex scripting routines. For example, the ‘Quality Control’ script constructs a large number of graphs and tables, some of which you may wish to re-use in your own custom Quality Control reports.

The GenePix Pro On-line Help (press “F1”) includes an extensive scripting tutorial in which the GenePix Pro Object Model is completely documented.

Find

Use the *Find* command to find features names and/or IDs anywhere in GenePix Pro. In the Image tab, *Find* highlights features that have the name or ID specified. 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 is a document that logs all file events that occur while GenePix Pro 3.0 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 into GenePix.

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.

You can optionally maintain different Lab Books for different users. If you have selected ‘Prompt for user name at startup’ in the *Options / Customize* dialog box, a unique Lab Book is used for each user.

On-line Help

The GenePix on-line Help uses the new Microsoft HTML Help interface, modeled on a Web browser, and should be effortless to use. It requires Internet Explorer 3.02 (but preferably version 4.0 or later) to be installed on your computer.



On the Help toolbar are two buttons – *Axon.com* and *Feedback* – linked directly to the Axon Instruments Web site. If your computer is networked, clicking on either of these buttons will

connect you directly to the Axon Home page, and the GenePix Feedback page, respectively, from within the Help window.

Sending Data to Other Applications

There are several types of data that GenePix creates that may be useful in other applications. For example, you may want to open your images in a desktop publishing application to create publication-quality figures. GenePix saves images in standard TIFF and JPEG format, which are readable in most current desktop applications.

Alternatively, you can copy the current image display to the clipboard and paste it directly into another application. When copying a displayed image from the Image tab, you can optionally copy the blocks of feature-indicators by toggling the *View Blocks* command.

You can also use the clipboard to copy an image or the data from the Histogram tab, and to copy the contents of the Results spreadsheet.

4. Summary

This is the end of the tutorial. We hope that you enjoy using GenePix Pro 3.0. Axon Instruments has put every effort into designing and constructing an application that will work with you to get your microarray tasks done efficiently

Remember to refer to the on-line Help and the rest of the User's Guide if you have any further questions about using GenePix Pro. If you encounter a problem that you can't solve, don't hesitate to contact Axon Technical Support.

Axon Instruments welcomes feedback on all its products. There is a Web page devoted exclusively to comments and suggestions on how to improve GenePix. You can reach it by clicking the *Feedback* button at the top of the on-line Help window. Otherwise, its URL is: http://www.axon.com/GenePix_Feedback.htm

Glossary

Array List

An array list is a file (*.gal) that contains block and gene information for the microarray, 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 gene names in the Array List. These names are then used in the various Feature Viewers, the Feature Pixel Plot dialog box and the Results tab.

Block

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

Complementary DNA (cDNA)

DNA that has been synthesized from messenger RNA (mRNA) by converting all mRNA bases into their complements (A to U, U to A, G to C, C to G). See test cDNA; reference cDNA.

Cy3, Cy5

Non-radioactive fluorescent dyes from Amersham Pharmacia Biotech (<http://www.apbiotech.com/>) that are widely used for labeling DNA in microarray experiments.

DNA chip

Originally used to describe microarrays created using semiconductor technology (*e.g.*, the GeneChip from Affymetrix, Inc.) but is now used to refer to DNA microarrays in general.

Dongle

The dongle is a software security device that attaches to the parallel or USB port of the computer. If GenePix does not detect the dongle you cannot save images, copy to the clipboard or export data. The dongle allows the port to be used for its usual purpose, *e.g.*, as a printer port.

Feature

A feature is a spot (typically of DNA) on a slide. The collection of such features is called a *microarray*. GenePix 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 GenePix calculates the pixel intensity and feature intensity of a feature on the microarray. A portion of the image outside the feature-indicator is used to calculate the feature background intensity.

Feature Background Intensity

The feature background intensity of a feature at a wavelength is the median intensity of the set of background pixels defined for that feature. The background pixels include all of the pixels within a circular region that surround the feature of interest. The circular region has a diameter of three times the diameter of the current feature-indicator. All pixels that are contained within a neighboring feature-indicator, or the feature-indicator of interest, are excluded. Using the median, rather than the mean, of background pixel intensities significantly reduces the effect of spurious pixels significantly contributing to the background.

Feature Intensity

The feature intensity is defined as 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

Added to a feature-indicator to mark some feature peculiarity, either positive or negative. In Feature mode, you can Flag features as 'Good' or 'Bad' by circling them, or by putting a cross through them, respectively. Flags are exported to the Results window. When exporting GenePix numerical data to spreadsheet programs like Microsoft Excel, Flags appear as '100'

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).

Hybridization

The process of joining two complementary strands of DNA, or one strand each of DNA and RNA, to form a double-stranded molecule.

Messenger RNA (mRNA)

RNA that is used to direct the protein synthesis that is part of gene expression.

Nudge

To move a single, or group, of feature-indicators within a block. Do this in Feature mode by selecting a feature-indicator and then moving it with the arrow keys.

Oligonucleotide

A short strand of DNA typically composed of up to 50 bases.

PCR

Polymerase Chain Reaction, a particular technique of making many copies of a DNA base sequence. The process is exponential, so that in a very short time more than 10^6 copies are made.

Pixel Intensity

The raw intensity of a pixel on a GenePix 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 GenePix are used to detect the photons that are emitted from the laser-excited fluorophores on the microarray.

Ratio Image

The ratio image is an RGB (Red-Green-Blue) overlay image. In this image, wavelength #1 (635 nm) is mapped to the green channel of the RGB image, and wavelength #2 (532 nm) is mapped to the red channel. Superimposing these two images onto each other results in a third, composite image, whose color is a blend of the red and green signals. Ratio images exported as JPEG or TIFF files are 24-bit RGB color.

Reference DNA

DNA from tissues of interest that is extracted at some reference time or in some reference state, *e.g.*, before the application of a drug. Reference DNA is mixed with *test* DNA and then hybridized with DNA spotted on a microscope slide to form a microarray.

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 voltage of the

PMT, which reduces its gain. Saturating pixels in GenePix are shown as white pixels in the raw wavelength images.

Spotted DNA

Known DNA that is spotted onto a microarray slide and used to identify unknown (*test* and *reference*) DNA that is hybridized with it.

Test DNA

DNA from tissues of interest that is mixed with *reference* DNA. The combination of test and reference DNA is then hybridized on a microscope slide with *spotted* DNA to form a microarray.

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