

Scanning using the Axon GenePix scanner

Time on the scanner is scheduled for both scanning and data extraction tasks at <http://calendar.oregonstate.edu/cgrb-genepix/>. To reserve time on the Axon GenePix Pro 4200A scanner Login and submit an event on the calendar. Contact [Caprice Rosato](#) if you are not yet an authorized user for this instrument.

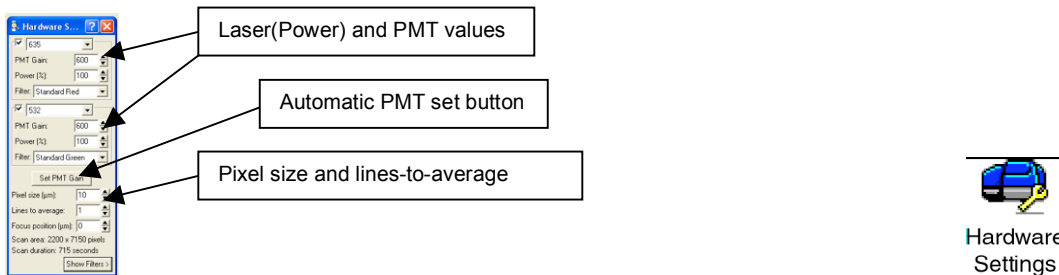
The scanner needs 20 minutes to warm up before you begin scanning. Turn on the scanner using the toggle switch (low center on the back of the instrument). Turn on (or reboot) the computer and sign in.

Double-click the GenePix software (6.0) icon on the desktop.

Place the array into the scanner

Before you can operate this independently you will need to work with [Caprice Rosato](#) for training. Open the cover and lift the stage lid. Holding the slide on the edges carefully place it on the stage with the array side down and the bottom of the array toward you. Gently move the seating arm to hold the slide in place. Lower the stage lid and push it down (listen for the click). Close the cover gently.

NOTE: If the slide is not seated onto the stage properly you risk breaking the slide, losing your data and possibly damaging the scanner.



Open the hardware settings using the icon on the right side of the main window. This window allows selection of the laser power, the PMT values, the pixel size and the lines to average. Double check that the filters are correct: Standard Red for 635 (=Cy5) and Standard Green for 532(=Cy3)!

Set the scanning parameters to minimize damage to the signal on the array, such as setting the laser power (e.g. 60) and the PMT (e.g. 450) for both channels (635 and 532). See note on top of next page.

Note: Laser power damages the fluorescent dyes so it should be kept low during the preliminary scans; the PMT (Photo Multiplier Tube) will affect how much background fluorescence is registered. Set the pixel size to 40 or 60 and the lines-to-average to 1. The lines to average tells the scanner how many times to go over the same area and average the readings. Often scans intended for data collection use lines-to-average = 2.



Do a Preview Scan to visualize the array location.

There are 2 ways for determining the setting to use for actual data collection. You can either use the scanner to make that determination through an Auto program or you can make decision by manual adjustments.

Note: If you have an expectation that one of the RNAs used for hybridization 'should be' more highly expressed than the other RNA you will need to balance the PMT setting using control features rather than the entire array. The control features are spots on the array where the abundance/expression of the RNA used in the hybridization is known to be equal for both RNAs used in the hybridization. This is outlined in #2-b below.

#1: Recommended for most applications: Have the scanner determine the PMT settings automatically for the best (most balanced) scan

The GenePix scanner can calculate the best PMT for a balanced scan of your hybridized slide. Before this automated feature will work you need to have both a Data Scan of the array and the .gal file loaded.



Click the Scan Area icon. Move the cursor to the top left corner of the array, click and drag to draw the box. Draw a box around the

perimeter of the array in the preview (ratio) image. Be sure to include a little bit of border outside of the array.

Perform a Data Scan



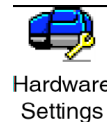
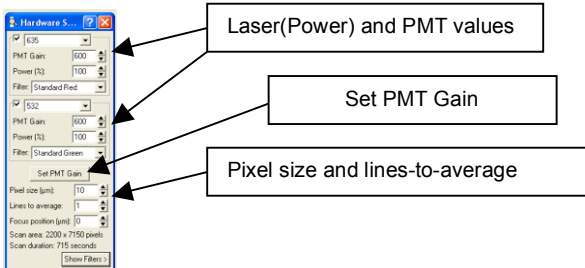
Keeping the same hardware settings as the Preview scan, do a 'data' scan. This will provide a template to use for the Auto feature of setting the PMT values to balance the entire array with minimal degradation of the signal on the array.



Using the File icon 'load the array list' which adds the .gal file for your array to the scanned image. Be sure you have the .gal file for your array available on the computer.



When the boxes from loading the .gal file appear open the Align Blocks menu and click 'Find Array'. The location of the .gal file image is the area used during the automated PMT calibration; check that the .gal file image overlays the area of the array that will be used for data collection. You can move the overlaid image by dragging or nudging (arrow keys). It is not critical that the circles line up with the features (yet).



On the Hardware Settings window set the laser power for both channels (635 and 532) to the power you wish to use for your scan (typically between 80-100). Set the pixels to the desired resolution (10 or 5 um). Click the "Set PMT Gain" button. Adjust the percent saturation (0.005% is the minimum, See p.41 in the User's Manual). Hit Start. **Note:** The higher the % saturation the more data from low abundant transcripts will be available at the expense of the data from highly abundant transcripts. Conversely, the lower the % saturation the more data from more highly abundant transcripts will be available

at the expense of low abundant transcripts. Features that are saturated (white) in the scanned image provide no useable data (their signal is above the upper range threshold). The data from a feature that has low signal can approach background values if the % saturation is set so that fewer features are white.

The scanner will perform several scans after which the recommended values will be shown as calculated by the scanner software. **Note:** the PMT values are calculated for the laser power you selected. If you change the laser power these PMT values may be wrong to produce a balanced scan. You can copy these into the Hardware Settings window or Hit Apply. You can then go to the Histogram tab and check that the balance is close to 1.0 (**Note:** a ratio that is >1.0 implies the balance between the red and green channels is off toward the red channel and conversely <1.0 implies greener than perfectly balanced).



If you are satisfied, initiate the Scan by clicking on the icon. You may also choose to have the instrument go directly from calculating the PMT to doing the actual scan. If you choose this option be sure you set the Pixel size and the lines-to-average to what you want before you click on 'set PMT Gain'. (Icon on right)

#2: To determine balance without the Automated calculation

The following steps describe how to set up the Hardware settings to perform a Preview scan. This will provide information so that you can choose hardware settings to use for a Data scan.

Set the pixel size to something large, e.g. 40 or 60. Set the PMT (e.g. 400) for both 635 and 532 channels. Set the laser power to the amount you want to use for your actual data collection scan (80-100). Set the lines-to-scan to 1.

During the Preview scan the PMT can be changed for each wavelength but the laser power cannot. Changing the PMT during the scan allows you to judge 'by eye' the balance over the array:

1. Allow the first (635=red) scan to finish without any manual intervention of the PMT.
2. During the second scan (green), allow a fraction (e.g. 1/4) of the array to be scanned before making any changes to the 532 PMT. If the image appears greener, lower the PMT by 10 or 20 (1 or 2

- clicks on the down arrow next to PMT value) and allow the scan to continue for the next section (e.g. 2nd ¼ of the array).
3. Continue adjusting the PMT for the green channel as necessary, but allow sufficient array area to be scanned at each PMT choice so that the value can be evaluated easily after the scan is completed. Conversely, if the image appears too red increase the PMT value.
 4. The goal is to have the balance of the channels to be equal (histogram indicating the ratio is = 1).
 5. Keep track of the PMT changes made (e.g. 1st ¼ at 400, 2nd ¼ at 380, 3rd ¼ at 390, 4th ¼ at 385).

Once the scan is complete zoom into each section of the array where the green channel was constant. **That means:** be sure that the area within the frame of the zoomed image is from the same 532 PMT value. Click on the histogram tab and look at the ratio value. Zoom back out to full image and zoom into the next area where the PMT value for 532 is constant but different than the previous zoom and look at the ratio in the histogram window. Continue down the array image until you can either find or guesstimate what the PMT gain value 'should be' to balance the 532 (green) image with the 635 (red) image.

When you have values for the hardware settings (Laser power, PMT gain values, pixel size and line-to-average draw a box around the array and do a data scan. (For review, see bottom of P.3 and top of P.4)

Click on the histogram tab and look at the balance. In a balanced array the ratio should be close to '1'. **Recall:** Greater than 1 indicates the red wavelength (635) is too strong whereas lower than 1 indicates the green wavelength (532) is too strong. If necessary make adjustments to the PMT and / or laser settings and redo the low resolution scan.

#2-b: To determine balance without the Automated calculation using specific features in the array (not the entire array)

If you are using control features to balance your array this will be similar to manually adjusting the PMT as described in #2 above except you will be using just the features where the balance of Cy5 to Cy3 is known to be equal. This is especially important when you have an expectation that one of your samples is regulated significantly differently than the other. **That typically means:** There are features

spotted on the array which are different from the organism of interest (e.g. Human genes on an Arabidopsis array, commercially available samples used as spike-ins (Alien spots by Stratagene)). Then an equal aliquot of the corresponding RNA can be added to each (Cy3 and Cy5) labeled sample during the labeling process. This way the actual RNA for each channel is known to be equal, and the array can be balanced using these features.



Scan Area

Click the Scan Area icon. Move the cursor to the top left corner of the features you will use to balance the scan, click and drag to draw the box. Draw a box around the perimeter of the control features in the preview (ratio) image. Check the hardware settings (laser power = 80-100, the pixel size = 5-10, lines-to-average = 1).



Data
Scan

Do a Data scan over the features. Check the balance in the histogram. Adjust the PMT value for the 635 and or 532 as necessary (see second **Note**, P.4).

Once you have determined the best settings for the balance of the control features, type them into the Hardware Settings and save the .gps file. Change the values in the Hardware settings to a low intensity level, e.g. 450 for PMT, both channels, 60 laser power for both, 60 pixels, 1 line-to-average. Do a Preview Scan. Then draw a box around the array for the Data Scan. Open the .gps you just saved. Do a Data Scan. To check the balance after the scan, zoom into the area of the control features and check the ratio in the Histogram window.

When you have determined the best settings for a given array, save the settings file (.gps) with an appropriate name (e.g. barcode.gps). Change the pixel size to 10 or 5 (change the lines-to-average to 2 if desired), do a Data Scan (for review see P.4). A scan can be stopped anytime. Remember, the lasers affect fluorescent molecules, and multiple scanning can lead to partial or complete degradation of signal. Resave the .gps file if you make any changes.

Archive your files

Note: Your image, settings (.gps) and report (.gpr) files can be copied onto your DVD and/or your flash drive at the GenePix computer. Some people store their files on their own servers (e.g. in the Science domain), and that is your choice. We recommend that you store the files on the cgrb server (IncomingData). Periodically we will remove the oldest files on the cgrb server, and it is therefore important for you to archive any files you wish to keep for future work.



Be sure to save the scanned image that you will use for data extraction in *multi-tiff* file format. Save the settings file (.gps) for each scan (e.g. YourSlideName.gps) where you can access them during analysis.

If you use BASE for archiving your arrays and / or preprocessing the data you will need to save your image files as 2 single-tiff files (one each, 635 and 532). Go to 'Save Image', choose 'single tiff format' and check that both 635 and 532 are checked. Save. Each single channel image will have the wavelength appended to the name to distinguish between them. The single-tiff images can be saved later as long as the multi-tiff image is still available.

When you are finished for the day, turn off the GenePix software. Remove your slide and turn off scanner.

Data extraction

Note: The scanner must be turned on for the software to recognize the correct wavelengths in the data extraction software. Turn on the scanner then boot (or restart) the computer for your analysis session. In the File menu open the multi-tiff image and the settings (.gps) file.



Open the scanned image that you will use for data extraction (*multi-tiff* file format). Open the settings file (.gps) for the image you have opened (e.g. YourSlideName.gps).



Use the Options icon (on right) to set the wavelength ratio for the analysis calculations. Click on the Analysis tab and look at the wavelength ratio [should be 635/532 if your control (reference) dye is Cy3 or 532/635 if your control (reference) dye is Cy5. See **Note** above.] Verify that the wavelength for your reference (control) dye is in the denominator of the ratio.

Note: Analysis in BASE uses Ch1/Ch2 to calculate ratios. This is done automatically when you attach a data file to a *hybridization* in BASE. Therefore it is critical to identify Ch1 and Ch2 by choosing the wavelength of your control (reference) RNA for the denominator in each raw data file, using the correct Ratio in GenePix. This will be part of the settings file (.gps) which you save and use when you do the analysis to generate the report file (.gpr) during data extraction.



Using this Align Blocks icon on the left side of the image window, under tools, choose one of the menu features (e.g. find array, find blocks, find features). Repeat for each of these commands (blocks, features) checking that each alignment is correct before proceeding to the next level of alignment. **That is:** if the alignment looks wrong after the 'find blocks' command, you will need to align the blocks manually before advancing to the 'find features' command.



Once the alignment of the features is approximately correct use the Zoom tool to zoom into the array and look at each feature to verify that the circles around the features are correct (position, size, etc.) If necessary, adjust the circles to best fit the features on the array. Circles can be moved by click-drag or using the arrow keys, and the size of the circles can be adjusted using the Control-Arrow keys (up = larger, down = smaller).

Note: The software has assigned an identity to each circle according to the .gal file. Be sure not to move a circle out of its intended position. This can be checked by putting the cursor over a circle and looking at the information at the left of the window, including block (= meta column + meta row), column, row.

Save the settings file (.gps) after you have aligned the features (or as you go) so that the changes you have made will be associated with this specific scan (image).

Do the Batch Analysis

Go to the Batch Analysis window (tab at top).

- If there are any files referenced in the working window (from someone's analysis session) highlight them and click on the 'Remove' button.
- Click on the 'Add' button; browse and find the multi-tiff file you wish to analyze. You can add more than one array, but be sure it is the multi-tiff image.
- Highlight each array in the working window of Batch Analysis (one at a time) and 'add .gps file'. Open the .gps file specific for the highlighted array.
- Once you have loaded in all of the image and corresponding .gps files, Hit the start analysis button. When the analysis is finished a .gpr file is created and saved in the same folder as the image file.
- Scripts for flagging features can be done in Batch analysis also, see GenePix Manual for details.