Application Note

Genomics

Axon Instruments, Inc.

PMT Adjustment in GenePix 4000B

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Last updated: August 15, 2001

Introduction

The GenePix 4000B uses two lasers to excite fluorophores such as Cy3 and Cy5, and a pair of high-sensitivity, low-noise photomultiplier tubes (PMTs) to detect emitted fluorescent light. Loosely defined, PMTs are optical components that convert incident photons into electrons via the photoelectric effect. When an incident photon impinges on the active surface of the PMT (the photocathode), a photoelectron is generated (Figure 1) [1, 2, 3]. This electron flows through a series of electron multipliers (dynodes) to the anode. The amount of current that flows from the anode is directly proportional to the amount of incident light at the photocathode.

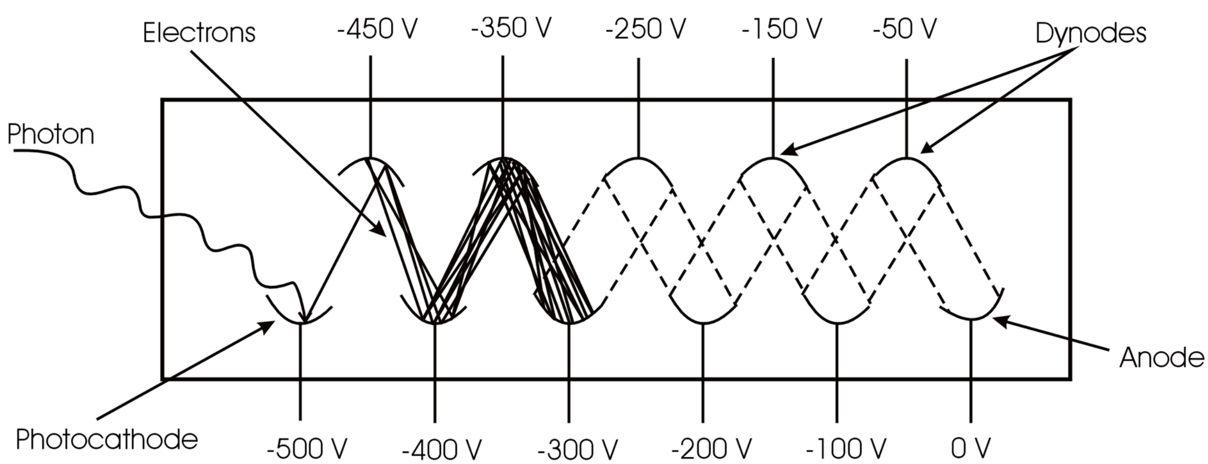


Figure 1. Diagram of a photomultiplier tube

The gain of a PMT depends on the voltage applied to the dynodes. The applied voltage accelerates the electrons to the dynode. The higher the PMT voltage, the more energy the electron has to strike the anode, and the greater the number electrons freed [1]. When the PMT gain (voltage) setting in the GenePix Pro software is increased, the sensitivity of the PMT is increased.

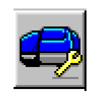
It is important to use optimal PMT gain. Though it is true that a higher gain yields a brighter image, a brighter image is not always a better thing. Increasing the gain of the PMT increases the noise as well as the signal intensity. If the gain is too high, the noise will increase more than the signal, and the signal-to-noise ratio becomes worse [1]. When the PMT gain is set too low, the process of converting photons to electrons is sub-optimal, and the signal-to-noise ratio is low. For the GenePix 4000B, the best signal-to-noise ratio can be achieved when the PMT gain is between 500 and 900. In general, we do not recommend using a PMT gain below 400. If the fluorescence intensity from the dye is saturating at full laser power, the laser power should be lowered rather than using an extremely low PMT voltage.

Three goals should be met to optimally set the PMTs:

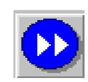
1. You must use the entire dynamic range of the GenePix scanner. GenePix digitizes PMT output to 16-bit, *i.e.*, 65,536 intensity levels. When the PMT gain is set too high, the signal will be saturated. If the PMT gain is set too low, the signal does not cover the entire dynamic range and effectively the signal has lower resolution.
2. Choose PMT gain settings that give the highest signal-to-noise ratio. Try to use a PMT gain between 500 and 900 if possible.
3. The fluorescence intensities of the features from both channels should be similar.

Steps for adjusting PMT gain:

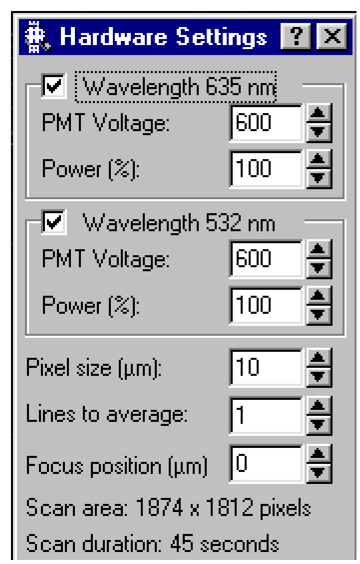
1. Load the microarray slide in the slide holder.
2. Click on the Hardware Settings button to open the Hardware Settings dialog box. It has options to change laser power and PMT voltage. Initially set PMT voltage to 600 and Power (%) to 100 for both channels.



3. Click on Preview Scan button for a pre-scan. This scan mode has lower resolution, thus is fast and causes less photobleaching.



4. As the features begin to appear, adjust PMT gain of both channels using the up and down arrows in the Hardware Settings dialog box. Increasing PMT gain will increase the intensity of the image. The following example is specific to Cy3 and Cy5, but the logic applies to other laser configurations. If the slide has control spots, adjust PMT voltage so that the control spot color is clearly yellow. If the slide does not have any control spots, adjust PMT voltage so that the spots are mostly yellow, some are red and some are green. This kind of adjustment is based on the assumption that when a large number of samples are studied, some samples will be up-regulated and some will be down-regulated. If all of the features are green, increase PMT voltage of red channel. If all of the features are red, increase PMT voltage of green channel. When PMT voltages are balanced, the color of most of the spots should be yellow (Figure 2). Decrease PMT voltage if the features become saturated (white).



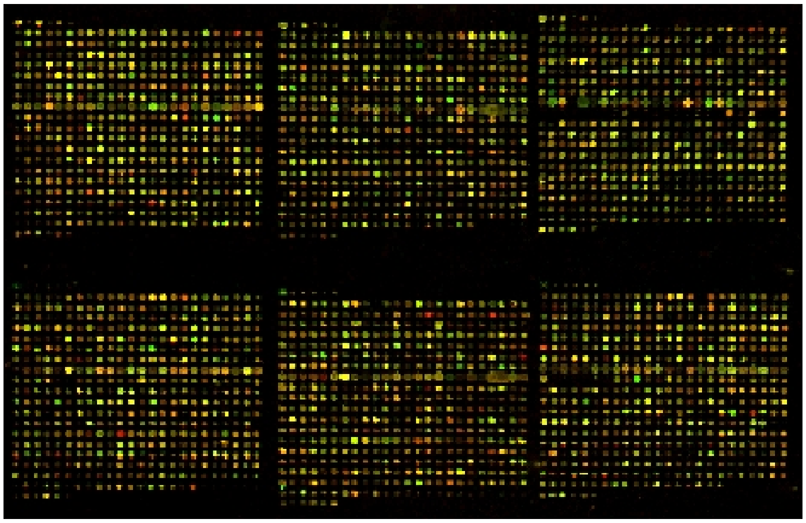
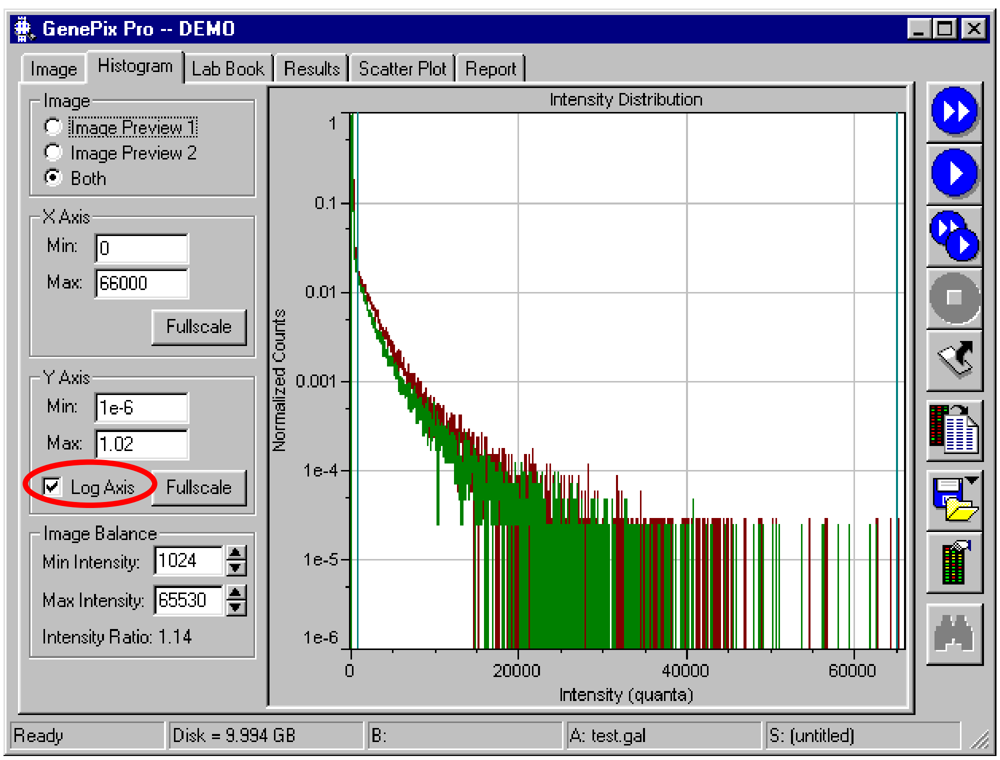


Figure 2. DNA array scanned in Preview Scan mode. Since the PMT is well balanced, most of the spots appear yellow.

5. Click on the “Histogram” tab located on the top of the screen to view the intensity histogram. The histogram displays the normalized count of pixels of all the intensities for both red and green channels. It is advisable to use the Log Axis, as the two traces are more clearly visible at the high-intensity end.



The Histogram indicates when the PMT gain is optimal. When the PMTs are correctly adjusted, the full dynamic range is utilized; a few pixels in the 65,500 intensity range are found in both channels. Once the intensities from both channels are balanced, the red and green traces overlap. In the histogram, the lower intensity portion of the curve is background, and the higher intensity portion of the curve is the signal from printed spots. When both the green and red curves are more or less overlapping in the high intensity region, the fluorescence intensities of the features in the green and red channels will be similar. If the PMT gain must be lowered to less than 400 to prevent having a large number of counts at the highest intensities, then the laser power should be decreased so that a higher PMT gain can be used.

6. Click on Image Tab to look at the scanned image. When the PMTs are well adjusted, the spots should be clearly visible, and most of the spots should be yellow, provided that the majority of the spots are control spots.
7. Stop Preview scan and start Data scan.

Discussion

Although we recommend that the PMTs be balanced so that the fluorescent intensities from both channels are similar, a significant error does not result if the two channels are not perfectly balanced. In reality, the signal intensities from both channels cannot be identical. This minor imbalance is corrected by software using normalization factor. GenePix Pro software calculates normalization factor based on the premise that the arithmetic mean of the ratios from every feature on a given microarray should be equal to 1 [4]. When the two channels are perfectly balanced, the normalization factor is 1.

We suggest that the PMTs be adjusted so that the normalization factor is close to 1 for two major reasons:

1. To optimize the dynamic range. If one channel uses a full dynamic range but the normalization factor is not close to 1, then the second channel is not using the best dynamic range.
2. To achieve the best visual effect of an image. When the two channels are well balanced, the normalization factor is close to 1. The green or red color of a spot shows up- or down-regulation of a gene.

In most cases, the analysis results are correct even though the normalization factor is lower or higher than 1. As long as the PMT voltage is not out of the recommended range or below what is necessary to properly detect a fluorescent signal, the integrity of the data is preserved. One needs only to use a normalization factor in the analysis process to calculate the correct ratios.

References

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