Application Note

Axon Instruments, Inc.

Genomics

Determining the Signal-to-Noise Ratio and Optimal Photomultiplier gain setting in the GenePix 4000B

Siobhan Pickett, M.S., Sean Carriedo, Ph.D. and Chang Wang, Ph.D.
Axon Instruments, Inc. 3280 Whipple Road, Union City, CA 94587

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Introduction

An important criterion for comparing the performance of array scanners is the quality of the images produced. One objective measure for evaluating image quality is the Signal-to-Noise ratio (SNR). The ability to distinguish a signal from the background image or the noise improves as the SNR increases. In addition to quantifying the quality of the scanned images, the SNR is used to define the detection limit and the optimal photomultiplier gain setting of a scanner.

Signal

The signal from the array scanner originates from photo-exciting a fluorophore . The photons emitted from the fluorophore are collected onto the surface of a photon-detecting device such as a photomultiplier tube (PMT)*. The signal (represented by S) generated by a photo-detecting device is a function of the number of emission photons (represented by N) contacting the detector and the detection system's quantum efficiency (represented by QE). Therefore, S = N x QE. It is important to remember that the quality of the signal depends on both biological and technical factors (based mainly upon the design considerations used in the array scanner). For instance, maximizing the collection of the emitted photons (N) is dependent upon designing efficient pathways not only for delivering the maximal amount of excitation light to the sample but also for directing the light emitted from the excited fluorophore onto the photon detector.

Noise

In all systems there are two noise components to consider, dark current noise and Shot noise. Additionally, sub-optimally designed systems may introduce noise in many different areas including the analog-to-digital conversion process or the amplification of the signal.

* Most laser-base arrray scanners use PMTs for detection. PMTs convert photons striking one end of the detector into electrons, and then amplify the electrons as they pass through to the other end. The amount of amplification (gain) can be adjusted by changing the voltage applied to the PMT.

Dark Current noise: Even in the absence of light input, the thermal emissions from any photon-detecting device can produce a low amount of noise (measured in electrons per second) that has been termed Dark Current noise. In the case of PMTs, Dark Current noise can originate from the photocathode and/or from current leaking through the dynodes of the PMT. The GenePix 4000B has been designed to reduce dark current noise to negligible levels through two approaches: 1) the PMTs chosen for use in the GenePix 4000B show very low dark current levels, and 2) the dwell time of the lasers on each pixel is very short resulting in a negligible number of dark current electrons generated for each pixel.

Shot noise: Shot or statistical noise is the only significant type of noise that needs to be considered in the GenePix 4000B. It is generated by the input of light and results from the variable nature of photons. With shot noise, it is important to realize that as the signal intensity increases the shot noise increases as the square root of the signal collected, so the SNR actually decreases as signal intensity increases.

Signal-to-Noise ratio

Signal-to-noise ratio is used in many signal-detection disciplines (radio, electronics, imaging, *etc.*) as a quantitative measure of the ability to resolve true signal from background noise. For fluorescent array imaging, SNR is commonly calculated as:

$$\frac{\text{Signal} - \text{Background}}{\text{Standard Deviation of Background}}$$

A higher SNR indicates higher signal over background noise; a signal-to-noise ratio of 3 is commonly considered the lower limit for accurate detection. Signal may be detected below this value, but the accuracy of quantitative measurements decreases significantly. Referring to the equation above, the SNR can be maximized by increasing signal, decreasing background, or decreasing the noise (*i.e.*, standard deviation of the background pixels).

In fluorescent arrays, the primary source of background signal is non-specific hybridization in the same plane of focus as the sample. The most effective way to increase the numerator in the SNR equation is to optimize hybridization and stringency wash conditions to minimize non-specific hybridization.

The denominator in the equation is determined by the "evenness" of the background signal. Contributing sources include biochemical factors such as probe purity and hybridization uniformity, as well as instrument factors such as stray photons and electronic noise inherent to all PMTs. Axon Instruments' core expertise is in ultra-low-noise signal amplification, and the GenePix 4000 Array Scanners are designed to minimize all sources of instrument noise.

Reflecting the importance of the SNR, GenePix Pro has a built-in script that automatically calculates the SNR for the scanned microarray, and draws a histogram of the SNR for each wavelength. The Signal-to-Noise ratio may also be calculated manually as outlined below. Either method is useful for comparing scanners from the same manufacturer or from different manufacturers.

1. Select a slide that is representative of your typical data slides.
2. Scan the slides at a PMT setting that gives the brightest image without any saturating pixels.
3. In Excel, divide the S-B value (*e.g.,* the "F532 Median-B532" column) by the N value (*e.g.*, the "B532 SD" column).

The type of PMT used in the GenePix 4000 Array Scanners has a wide dynamic range, so it detects and amplifies both strong specific signal on arrayed spots, and low-level background fluorescence, which is primarily due to non-specific hybridization. Simply increasing the gain to the maximum setting may not produce the optimum SNR. Thus, the optimal PMT setting must be determined empirically. The first step is to make a dilution series of the probes to be used. Next, the relationship between the SNR and PMT gain is determined. From these data the optimal PMT settings for the fluorescent probes is obtained. Data presented below illustrates the relationship between a wide range of PMT voltages and the concentration for the commonly used Cy3 and Cy5 probes.

1. SNR as a Function of PMT Gain for a Single Dye Concentration.

The following experiments indicate that the signal-to-noise ratio continues to improve up to about 500 V. PMT gain above 500 V will give equal SNR for dye concentrations at the middle of the concentration range (**Figure 1A**), as well as near the detection limit (**Figure 1B**).

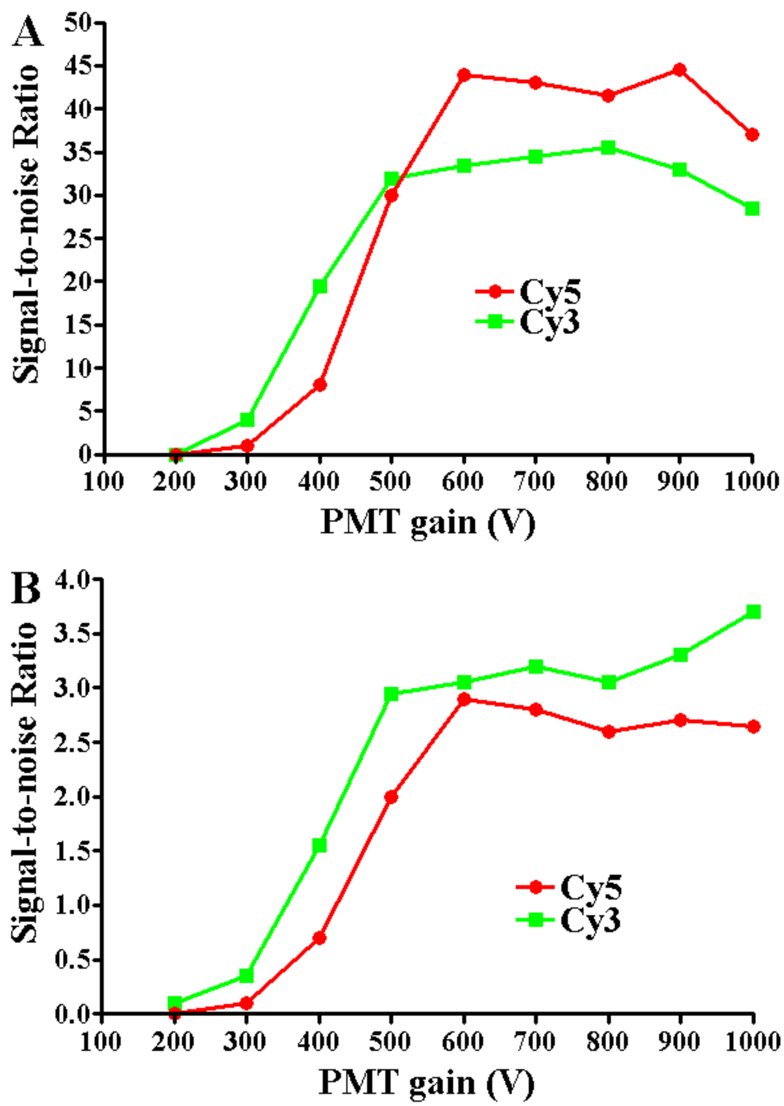

Figure 1: SNR for both the Cy5 (Red line) and Cy3 (Green line) was measured over the full range of PMT voltages for a single spot in the middle of a dilution series (***A***, below saturation at 1000 V), and near the detection limit (***B***, defined as SNR = 3).

2. Effect of PMT Gain Over Full Range of Fluorescent Concentrations.
A dilution series of dyes Cy3 (**Figure 2A**) and Cy5 (**Figure 2B**), spanning six orders of magnitude, were scanned over the entire range of PMT voltages. At any dye concentration, the SNR improves from 200 V to about 500 V. Above 500 V, the highest spots in this dilution series begin to saturate, so the linear range is reduced at the top end. There is no difference in detection limit for either dye at a PMT gain between 500 V and 900 V.

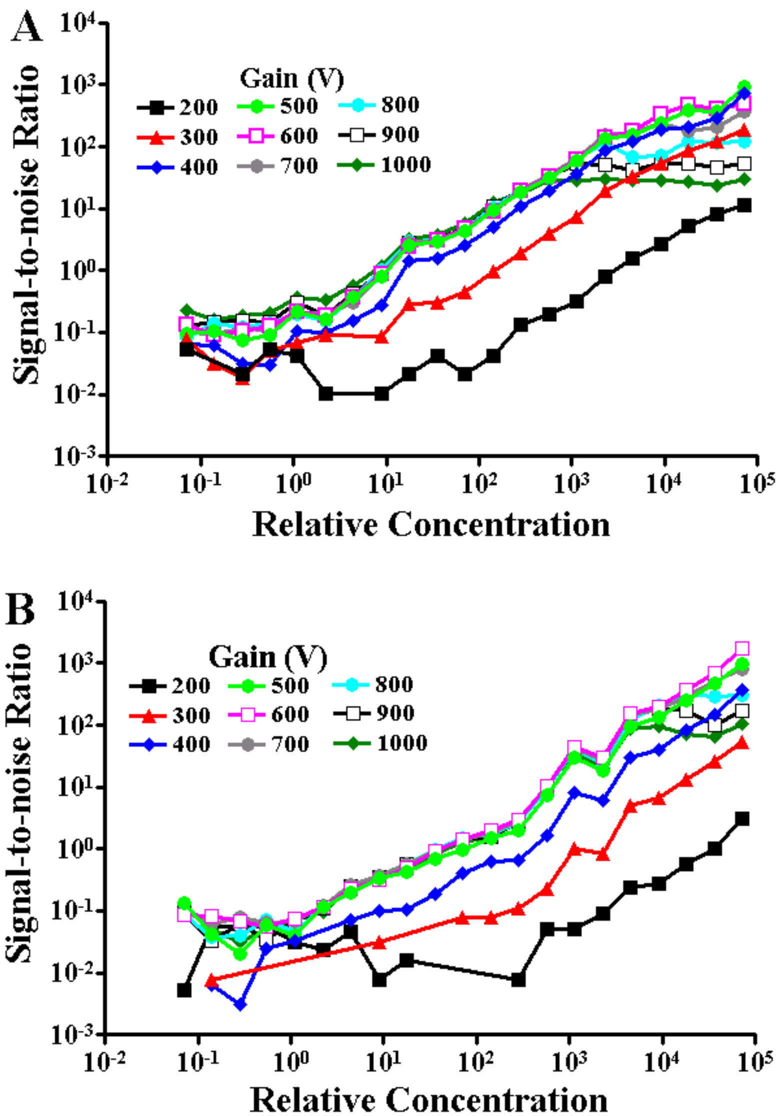

Figure 2: SNR over a full range of PMT voltages for both the Cy3 (***A***) and Cy5 (***B***) was measured for a dilution series spanning six orders of magnitude.

Conclusion

For a wide range of Cy3 and Cy5 conecntrations, the optimum PMT gain on the GenePix 4000 Array Scanners is anywhere in the range from 500 V to 900 V, as long as the brightest signals are below saturation.

Further reading

Basarsky, T., Verdnik, D. Zhai, J.Y. and Wellis, D. Overview of a Microarray Scanner: Design Essentials for an Integrated Acquisition and Analysis Platform. pp 265-284. In: Microarray Biochip Technology. M. Schena (Ed.), BioTechniques Books, Natick, MA. (2000)