A Method for Quantifying the Performance of a DNA Microarray Scanner

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Introduction

The microarray scanner plays a pivotal role in the DNA microarray processing workflow and can profoundly affect the quality and reliability of microarray data. Since important decisions are often based on microarray scanning results, it is critical to understand the magnitude of the error contribution of each step in the microarray processing workflow compared to the total error of the microarray analysis process. For lack of a simple method for assessing scanner performance, the microarray scanner is frequently ignored as a source of experimental error.

Typical sources of error from a microarray scanner include: noise in the background light, non-uniformity of the scan field, variations in laser brightness and detector gain, and spectral cross talk between dye channels. These parameters are sometimes difficult for the end-user to measure individually.

Part I of this paper describes a simple and effective method for quantifying scanner performance based on standard microarray metrics related to scanner noise, including detection limit, signal to noise (S/N), and the standard deviation of the log (Ratio). This method is available to any researcher interested in assessing the performance of any microarray scanner or set of scanners. Those who already have a scanner will find this method useful for evaluating the quality of experimental results. For those who are considering the purchase of a microarray scanner, it can help in making an informed decision

Part II of this paper uses the data set generated in Part I to evaluate the total experimental error introduced by the integrated Agilent microarray-based Gene Expression system. This includes the microarray scanner's contributions to error as well as error introduced by microarray printing, sample preparation and labeling, hybridization, washing, and feature extraction.

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Part I:
Evaluating scanner performance

The microarray processing workflow involves a number of steps, each of which contributes differing amounts of error to the final measurement.
These steps include:

- Microarray manufacture
- Sample preparation and labeling
- Microarray hybridization
- Microarray scanning and imaging
- Data extraction and analysis

Taken together, the error contributed by each of these steps adds up to the total experimental error budget. The objective of this study was to develop a simple method for quantifying the scanner's contribution to the total error budget independent of error contributed by other steps in the microarray processing workflow.

Materials

To measure microarray scanner performance independent of other system components, it is necessary to compare images of identical samples taken under identical conditions. To meet this requirement as closely as possible, the following materials and equipment were used in this study.

Three Scanners – The Agilent dual laser DNA Microarray Scanner (Part Number G2565BA) and two instruments labeled Scanner Y and Scanner Z.

Three "nominally" identical oligo microarrays – Each microarray used in the study was an Agilent Human Oligo 1A Microarray Kit (Cat. Number G4110A) and was printed on 1-inch by 3-inch (25.4mm x 76.2mm) glass slides. All three were from the same print wafer and hybridized in the same batch using the Agilent in situ Hybridization Kit Plus (P/N 5184-3568) according to the recommended procedure. Each microarray included 19,777 features of 60-mer oligos. Each of the microarrays also included 100 probes with 10 replicates.

Target materials – All targets were prepared using the Agilent Linear Amplification Kit (Product Number G2554A) and consisted of cRNA generated from Clontech Universal Reference labeled with cyanine 3, and from placenta labeled with cyanine 5.

Procedure

All three microarrays were scanned eight times on each scanner at 10 µm resolution, for a total of 72 scans. To eliminate bias due to ordering, scanner order was varied, as shown in the table below.

	1st Scanner	2nd Scanner	3rd Scanner
Microarray 1	Agilent	Brand Y	Brand Z
Microarray 2	Brand Y	Agilent	Brand Z
Microarray 3	Brand Z	Brand Y	Agilent

How much does the microarray scanner contribute to the error budget of a DNA microarray experiment?

Figure 1. Comparison of scans

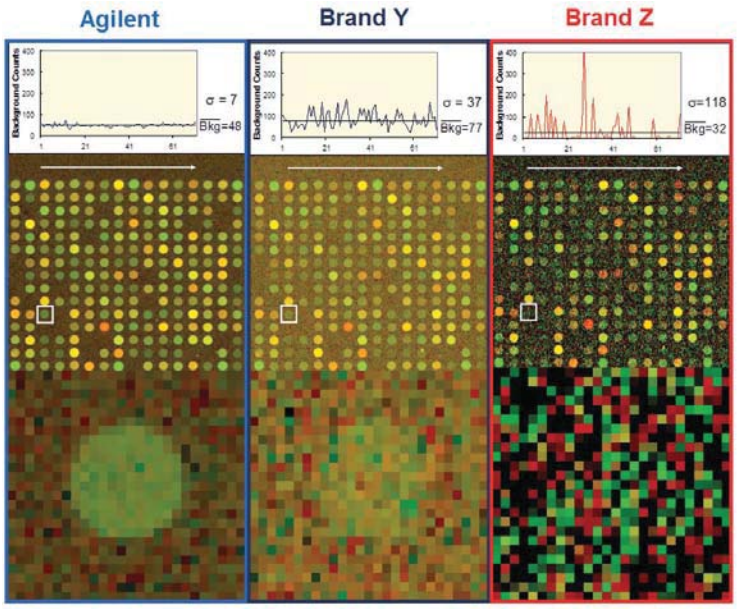


Image quality and scanner noise

Figure 1 displays the two-color scan image of the same area of the same microarray on the three commercial scanners used in this study. The color scale for all images is logarithmic with black and brightest pixels representing those pixels outside of one percent and 99 percent of the distribution, respectively. The gain for the Brand Y and Brand Z scanners was equalized using a calibration slide. The gain on the Brand Y scanner was set successfully within approximately three percent of the Agilent DNA Microarray scanner (G2565BA). The Brand Z scanner's gain was approximately 1.2 times the Agilent scanner's gain.

For each scanner, the middle panel shows an approximately 3x3 mm section of the microarray with approximately 120 µm features. Notice the increasing noise visible when comparing Agilent to Brand Y and then Brand Z. In the upper panel, the red background counts are plotted versus pixel column along with the mean and standard deviation (σ) of the background. The white arrow shows the approximate location of the background shown. The difference in the noise on the background is apparent. (Note: For Brand Z, the stated σ = 118 is 2.2x larger than the measured σ because 45 percent of the background pixels are censored at zero counts.) In the lower panel, the feature outlined with the white box is expanded. Notice that the feature is clearly distinguished on the Agilent scan; can be made out with difficulty on the Brand Y scan; and is not detectable in the Brand Z scan. On the Agilent scanner, the signal level of the outlined feature shown is eight counts and 58 counts (over background) in the red and green channels, respectively.

Results

After measurements were complete, the effects of the individual parameters on the scanner performance were used to quantify the performance of the individual scanners. Results for each scanner are summarized in the table below.

	Agilent	Scanner Y	Scanner Z
Detection limit (cpsm)*	0.07	0.33	1.4
Features below detection limit (in either channel)	158	807	8154
S/N (for signals brighter than 5000 counts)	149	51	19
(log(Ratio)) (most common)	0.007	0.03	0.11

*The number stated for the detection limit is the average of the red and green channel detection limits. To convert the detection limit measurement from a signal level to a dye density (chromophores per square micron [cpsm]), we used a calibration slide with a known dye density. To determine the average S/N, we included data points from both the red and green channels provided that the signal in that channel was over 5,000 counts.

Detection limit

The detection limit of the scanner is a measure of the dimmest feature whose signal (S) can be distinguished from the noise (N). By way of convention, all features whose S/N<3 are considered below the detection limit. The detection limit for each color is the median S for those features for which 2.8<S/N<3.2.

Standard deviation (σ) of log(Ratio)

The standard deviation (σ) of log(Ratio) is a measure of the variability that the scanner introduces into the log (red signal/green signal). The σ(log(Ratio)) for every feature is defined as the σ of the log(Ratio) for that feature among all eight repeated scans of that microarray. To simplify, we combine the σ(log(Ratio)) of the three microarrays by averaging for every feature the σ(log(Ratio)) of that feature on all three microarrays.

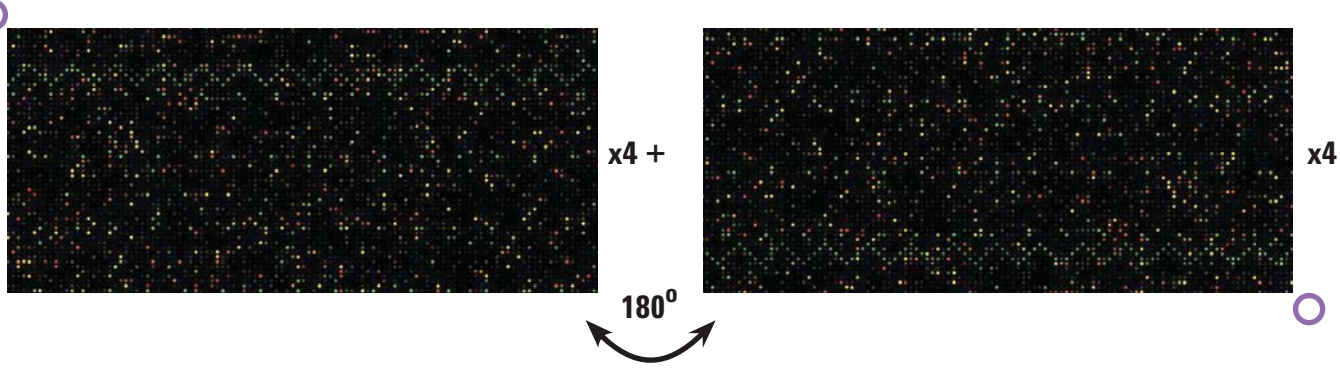
Scanner S/N and detection limit

By plotting the average S/N for all green features versus the average feature signal for all green features, it is possible to see how many features fall below the detection limit (S/N=3) for all three scanners. The differences between the three scanners can be seen in Figure 3. The Agilent scanner has 158 features below the detection limit in either channel, Brand Y has 807 undetectable features, and Brand Z has 8,154 undetectable features. There are 19,777 features on the microarray.

To include the effects of uncertainties caused by variation in gain due to spatial position in the scan area, each microarray was scanned four times, rotated 180 degrees and scanned the remaining four times (see Figure 2). Therefore, if the signal of a given feature varies due to its position in the scanner's field of view, this uncertainty is included in the measurement. A cyanine 3 plus cyanine 5 calibration slide was used to equalize gain setting of all the scanners. Each set of eight repeated scans for one microarray on one scanner took approximately one hour.

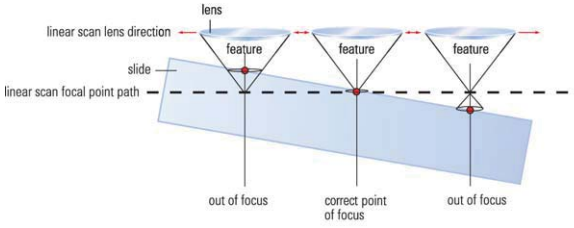
Data from all 72 scans were extracted using Agilent Feature Extraction software version 6.1.1, using Agilent internal tools to allow processing of non-Agilent scan images. The data were analyzed and plotted using Microsoft® Excel and Spotfire DecisionSite.

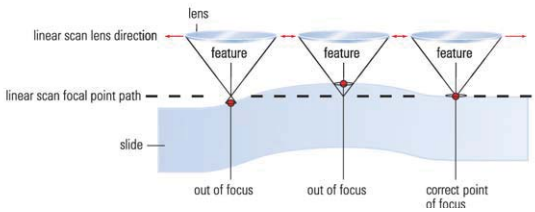
Figure 2. Scan rotation
Microarrays were scanned four times in forward direction and four times in reverse to include gain variations due to spatial position.



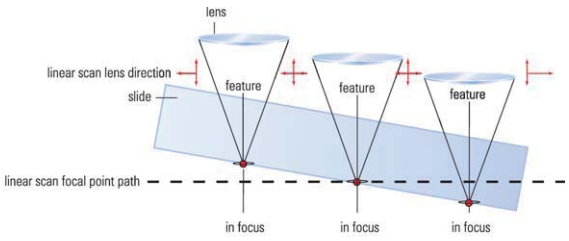
Agilent's industry-first dynamic autofocus
Agilent's SureScan technology includes a unique dynamic autofocus feature that continually focuses and refocuses during the glass slide scanning process. Compared to other commercial scanners which only focus on a few discrete spots on a slide to compensate for glass abberations, Agilent's dynamic autofocus adjusts 1000's of times to gracefully flow with any curvature, warpage or other common glass slide imperfections. The end result is highly sensitive and confident results researchers can trust for their downstream data analyses.

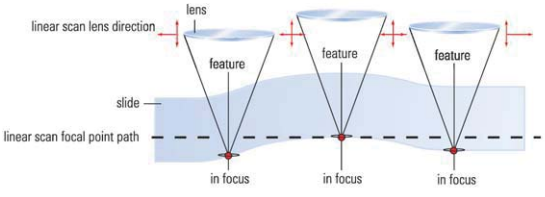
Other commercial scanners
Single point of focus can cause microarray features (spots) to be out of focus–leading to questionable data





Agilent Microarray scanner
Dynamic autofocus continually adjusts to keep the feature (spot) in focus for more confident microarray data





Data analysis

After data from the scanned images were extracted, they were used to calculate the following performance metrics for each scanner. These metrics are a measure of the performance of the scanner, independent from the rest of the microarray system.

All of the metrics defined below include the variation from the scanner and Feature Extraction software only. This is done by defining the noise or variability for all of the metrics based on the amount of change in the signal for each feature from one scan of the same microarray to the next scan in the same scanner. In other words, the variations are due to scan-to-scan non-reproducibility and therefore do not depend on any irregularities in the microarrays or their processing. In Part I of this study, we are not considering variations in microarray or feature replicates; we are considering variations in scan image replicates. The extent that a given feature on a given microarray doesn't yield the same signal from one scan to the next, is most likely due to a scanner-induced variability. The metrics also include variations due to the feature extraction SW algorithm, but the variability introduced by the feature extraction algorithms was found to be much smaller than the amount introduced by any of the three scanners.

Signal/Noise (S/N)

Image quality is determined by the S/N ratio. S/N is the ratio of the intensity of the desired signal to the amplitude of noise signals at a given point in time. The ability to distinguish a signal from the background improves as the S/N increases. In general, the higher the S/N, the better the image quality.

The signal for each feature is defined as the average of the background-subtracted signal for that feature averaged over all eight scans of that microarray.

$$Signal(Feature_x) \equiv \sum_{scan_1}^{scan_8} \frac{Signal(x)_{scan_i} - Background(x)_{scan_i}}{8}$$

The noise for each feature is defined as the standard deviation of the background-subtracted signal of that feature among all eight repeated scans of that microarray.

$$Noise(Feature_X) \equiv \sqrt{\frac{1}{8-1} \sum_{scan_1}^{scan_8} \left[(BkgSubtractedSignal_{scan_i} - \overline{BkgSubtractedSignal})^2 \right]}$$

There is an S/N value for every feature, for both colors, on every microarray and on each scanner. To simplify the calculation, the S/N value for each feature for both colors in all three scanners was combined by averaging the S/N of that feature on all three microarrays. This produced an S/N value for all 19,777 features for both colors on all three microarrays for each scanner.

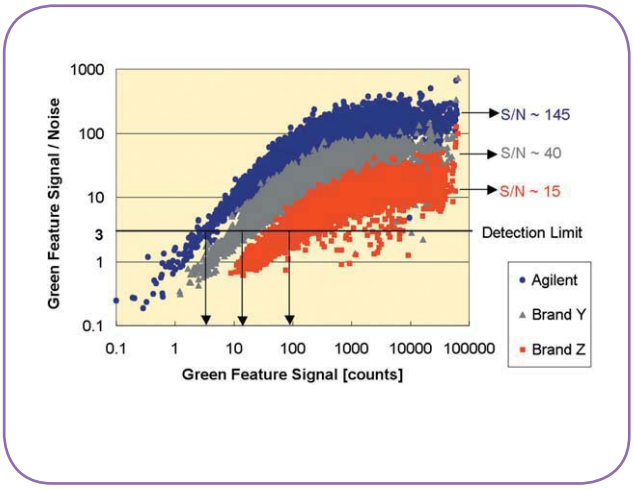
Detection limit and noise

It is the level of the noise on the background, not the average background, that determines the detection limit of a scanner. To determine how bright a feature must be to be detected, it is necessary to know the amount of noise on the background. Figure 4 shows the relationship between detection limit and the noise of the background for all three scanners. The detection limit of the scanner is set by the background noise, but the quality of the data above the detection limit is determined by the S/N of the brighter features. This S/N is inverse to the uncertainty of the measurement. As shown in Figure 4, there were clear differences in this important metric between scanners used in this study.

Sources of variation in S/N

There are two broad categories of variation, which decrease the effective S/N. The first is scan field non-uniformity, or variation in the scanner's effective sensitivity depending on where in the image the signal is generated. The second, more familiar source, is simple random variation in the effective sensitivity (i.e. from laser power or detector gain fluctuations). Together, these two types of uncertainty determine the effective S/N, whose measured value was presented above. To more clearly visualize the differences between these two sources, and understand their relative importance, it is useful to measure a slide that is, to the greatest extent possible, uniformly coated to produce a uniform signal.

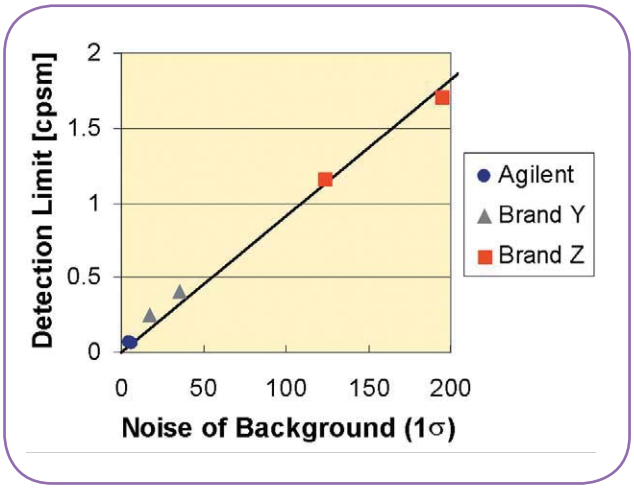
Figure 3.



Green feature S/N versus green feature signal for the three commercial microarray scanners

For high signal counts, the S/N approaches a constant level. The average S/N for feature signals (counts) above 5,000 is shown at right. The arrows mark the point where each scanner's data crosses the detection limit (S/N=3). The data are similar in the red channel.

Figure 4.



Detection limit (chromophores per square micron) vs. background noise (1σ)

For the three scanners measured, and both colors, the detection limit is roughly linear with increases in background noise. To determine the detection limit in chromophores/μm^2, the scanner's sensitivities were measured at the gain settings established for this study. The average sensitivity is approximately 50 counts per pixel/cpsm. The detection limit in these scanners varies by more than a factor of approximately 20x. Notice that while the average background levels for these three scanners vary by only approximately 3x, the noise on the background varies by approximately 30x.

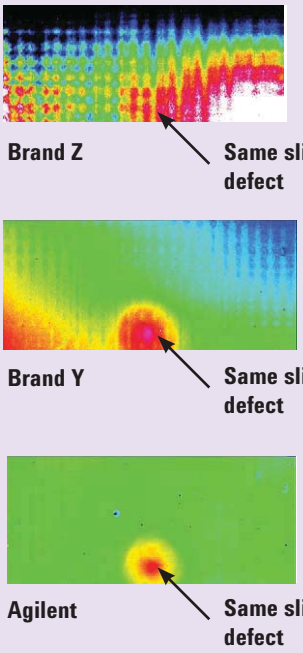
Scan field non-uniformity

In this study, we first consider the effect of scan field non-uniformity. This can be understood as the degree to which the same dye density registers a different signal depending on where in the scan image it is measured. The extent of this non-uniformity varies for different scanners. To the extent that a scanner's field is non-uniform, it will make the uniform slide appear non-uniform in the scan image. This effect is demonstrated graphically in Figure 5. These images were obtained from a single, uniformly coated test slide scanned on all three scanners (single color). The color scale was set the same for all three scanners. Black represents –10 percent of the mean; white is +10 percent.

Random noise

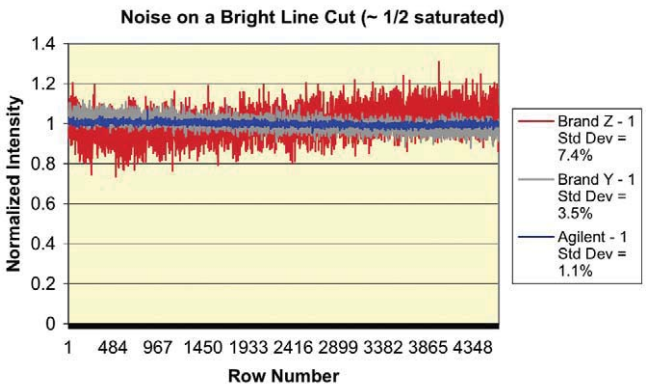
Separate from uncertainty introduced by scan field non-uniformity, every scanner adds random noise to the measurement from many sources, including laser noise, detector noise, noise due to the processing electronics, etc. For different scanners, the magnitude of this noise varies. To see this noise contribution, we considered the signal along a portion of a single scan row of the image. As seen in Figure 6, the standard deviation of the measured signal from the Agilent scanner is lower than that of the other two scanners. Taken together, the superior uniformity and low noise of the Agilent scanner delivers its superior S/N characteristics for the brighter features on the microarray.

Figure 5.



Uniformity of scan field for all three commercial microarray scanners
The differences between the three images are clearly visible. The Agilent scan field is relatively uniform across the entire slide and varies less than 2 percent (standard deviation of all pixel values). The brighter region in the bottom center of the image is a slide defect. This was verified by rotating the slide and confirming that the bright region rotated as well. The Brand Y scan image varies in a near linear fashion from lower left to upper right, with a pixel standard deviation of more than 4 percent. The Brand Z image varies more than 10 percent across the slide. If a feature will yield a signal that varies by 5 or 10 percent depending on where it is placed on the microarray, the certainty of the measurement is affected.

Figure 6.

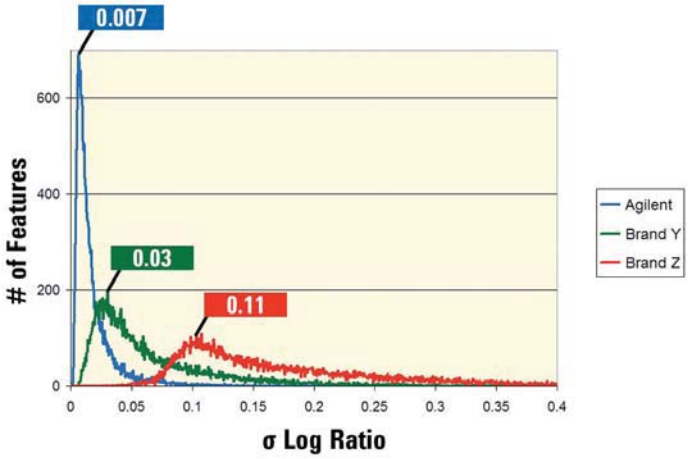


Random noise on bright line cut
The standard deviation of the measured signal from the Agilent microarray scanner is lower than that of the other two scanners.

Variability of log(Ratio) and differential expression

In gene expression experiments, the log of the ratio of red signal/green signal (log(Ratio)) is the preferred metric to calculate signal ratios for a given feature. Since this is the form in which most users state their data, it is useful to determine the effective error bars on the data expressed in this form. The standard deviation (σ) of the log(Ratio) measures the dispersion of log(Ratio) values from the scanner around the mean and places a lower limit on the minimum detectable differential expression in a microarray experiment. Figure 7 shows a distribution of the σ(log(Ratio)) values for the three scanners evaluated in this study. This figure illustrates the impact of the scanner's variability on the experimental error bars of the log(Ratio). Remember that this variability is defined by scan-to-scan reproducibility and, therefore, measures the scanner contribution to the experimental uncertainty. It is often thought that the scanner-induced variability is much smaller than the total uncertainty of the measurement, and thus is not important. Of course, this is the ideal and is not achieved equally well by all scanners.

Figure 7.

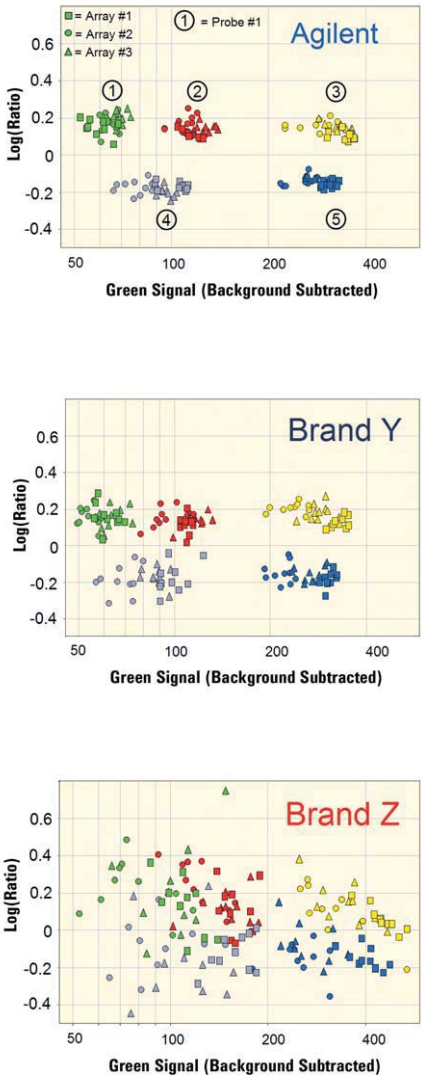


Histogram of the feature standard deviation of log(Ratio)

The most common σ(log(Ratio)) for the Agilent microarray scanner is 0.007. This allows a 1.05x differentially expressed gene to be measured with a confidence interval of 3σ. Brand Y has a σ(log(Ratio)) mode of 0.03, or approximately 1.23x minimum differential expression. Brand Z has a σ(log(Ratio)) mode equaling 0.11, or approximately 2.14x minimum differential expression. It is often thought that the scanner-induced variability is much smaller than the total uncertainty of the measurement, and thus is not important. Of course, this is the ideal to be strived for; but, as seen here, not achieved equally well by all scanners.

Part II:
Evaluating system uncertainty

Figure 8. Inter-array variability



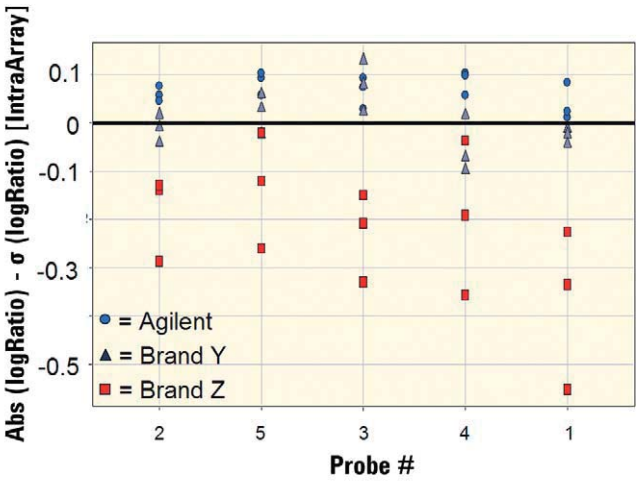
Data from five probe sequences with 10x replicates on each of the three microarrays with a log(Ratio) within 0.2 of zero were plotted to see if they were differentially expressed with 3σ confidence. These probes were also chosen because they have a low signal level (i.e., a few hundred counts). Effectively, this subset probes were chosen because they would be the most challenging to accurately measure as differentially expressed. The spread of the log(Ratio) is shown for all five probes on each scanner separately. The Agilent microarray scanner's data cluster most consistently with an average σ(log(Ratio)) of 0.036; Brand Y and Brand Z have average σ's of 0.054 and 0.133, respectively.

The method presented in Part I of this paper quantifies the microarray scanner's contribution to the total experimental error using repeated measurements of each feature. This was done by looking at variations in multiple scans. In the following experiment, uncertainty of the entire microarray-based system—including contributions from the microarray, labeling, hybridization and data extraction—is determined using data extracted from replicate probes (inter- and intra-array) from a single scan.

The three "nominally identical" microarrays used in this study each had 100 probes that were replicated in 10 positions on the microarray. The group of figures shown below uses data extracted for these probes to show the effects of different s microarray scanners on the total experimental error of a microarray experiment. These data were obtained from a single scan of each microarray on each scanner (i.e., data from the first scan described in the Part I procedure). Consequently, slide rotation was not used in this experiment to include the effects of uncertainties caused by scan field non-uniformity. Instead, we consider these effects for the entire experimental system by looking at the variability of data from replicate probes in different positions on the microarray, and between nominally identical microarrays. For a perfect experiment (i.e., zero uncertainties), all 30 replicates (10 each from three microarrays) should all report the same log(Ratio).

These results show that the Agilent microarray-based Gene Expression solution (including microarray printing and hybridization, scanning and feature extraction) generates data with high enough quality that even relatively dim signals (approximately 5 cpsm) are measured accurately enough to detect differential expression as low as 1.5x. For brighter signals, differential expression as small as 1.3x should be measurable. This is a very high level of performance considering that it includes all of the error introduced by all steps in the Agilent microarray workflow.

Figure 9. Intra-array variability



The combined plot above shows the absolute value of the average log(Ratio) for each of the five replicate probes on each microarray minus three times the standard deviation. When this value is above zero, we can call the probe differentially expressed to a confidence of better than 3 . This is equivalent to stating that the signal to noise ratio of this measurement of the log(Ratio) is higher than three. The Agilent microarray scanner sees all 15 data points as significantly differentially expressed. Brand Y identifies only 8 of 15. Brand Z identifies none of the 15 probes as significantly differentially expressed.

Discussion

As the quality and consistency of microarray analysis platforms continues to improve, it becomes increasingly important to isolate and quantify sources of error in the microarray processing workflow that may affect an experiment. This information enables you to place greater confidence in the accuracy of you results and decisions that are based upon those results.

The method described in Part I of this study for quantifying DNA microarray scanning performance offers a simple and effective way of assessing the impact of scanner noise on experimental uncertainty. Using standard microarray metrics, namely S/N, Detection limit and log(Ratio), this method was used successfully to quantify the performance of three different commercial microarray scanners. The results show that these performance metrics varied significantly among the three scanners and had a measurable and dramatic impact on image quality.

Part II of this study described the measurement of the total experimental error introduced by the complete Agilent microarray system, including the scanner, microarray, sample preparation, labeling, hybridization, and data extraction. The results clearly demonstrate that even when all sources were included, variability with the Agilent microarray-based Gene Expression system was minimal. The results of Part II also underscored the variability in performance among the three microarray scanners used in the study.

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