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Gene expression

Scanning microarrays at multiple intensities enhances discovery of differentially expressed genes

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ABSTRACT

Motivation: Scanning parameters are often overlooked when optimizing microarray experiments. A scanning approach that extends the dynamic data range by acquiring multiple scans of different intensities has been developed. **Results:** Data from each of three scan intensities (low, medium, high) were analyzed separately using multiple scan and linear regression approaches to identify and compare the sets of genes that exhibit statistically significant differential expression. In the multiple scan approach only one-third of the differentially expressed genes were shared among the three intensities, and each scan intensity identified unique sets of differentially expressed genes. The set of differentially expressed genes from any one scan amounted to <70% of the total number of genes identified in at least one scan. The average signal intensity of genes that exhibited statistically significant changes in expression was highest for the low-intensity scan and lowest for the high-intensity scan, suggesting that low-intensity scans may be best for detecting expression differences in high-signal genes, while high-intensity scans may be best for detecting expression differences in low-signal genes. Comparison of the differentially expressed genes identified in the multiple scan and linear regression approaches revealed that the multiple scan approach effectively identifies a subset of statistically significant genes that linear regression approach is unable to identify. Quantitative RT–PCR (qRT–PCR) tests demonstrated that statistically significant differences identified at all three scan intensities can be verified.

Availability: The data presented can be viewed at http://www.ncbi.nlm.nih.gov/geo/ under GEO accession no. GSE3017.

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Supplementary information: Data from these experiments can be viewed at http://www.plantgenomics.iastate.edu/microarray/data/

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INTRODUCTION

DNA microarrays simultaneously examine the relative abundances of thousands of transcripts in two RNA samples (Schena *et al*., 1995). Microarray experiments can be divided into seven steps. Much has been published regarding experimental design (Churchill, 2002; Kerr, 2003; Kerr and Churchill, 2001; Simon and Dobbin, 2003; Yang and Speed, 2002), array production (Diehl *et al*., 2001; Rickman *et al*., 2003; Taylor *et al*., 2003), RNA isolation and amplification (Baugh *et al*., 2001; Luo *et al*., 1999; Naderi *et al*., 2004; Pabon *et al*., 2001; Van Gelder *et al*., 1990; Wilson *et al*., 2004), labeling and hybridization considerations (Heller *et al*., 1997; Yue *et al*., 2001), and downstream data analyses (Leung and Cavalieri, 2003; Quackenbush, 2002; Slonim, 2002; Wolfinger *et al*., 2001). However, an often-overlooked aspect of microarray experiments is post-hybridization data acquisition. Although the seminal microarray publication (Schena *et al*., 1995) described a data acquisition strategy using two different laser settings, this approach is not used in typical microarray protocols. Instead, most protocols recommend scanning one time per channel at settings that minimize the number of saturated spots (Hegde *et al*., 2000; Leung and Cavalieri, 2003). While this approach captures a subset of the statistically significant differences, it potentially excludes genes on the basis of signal intensity. Duggan *et al*. (1999) reported that one of the limiting factors in microarray experiments is signal detection for low-signal spots. Indeed, scan intensities necessary for preventing saturation of high-signal genes may prove inadequate for the detection of differential expression in low-signal genes.

Procedures that correct saturated spots have been explored by Dudley *et al*. (2002) and Dodd *et al*. (2004). Although both procedures extend the dynamic range of the acquired data, differences in gene expression may be undetectable when the data are analyzed as a single set. An alternative approach to obtain more gene expression information is to independently analyze the datasets collected at different scan settings, and subsequently combine all the analyses. This approach was applied to an experiment aimed at identifying differences in transcript abundance in developing

maize anthers. The tassel, which is the male reproductive structure of maize, bears spikelets that contain anthers that proceed through a series of morphologically defined developmental stages and ultimately produce pollen.

Here we describe the analysis of multiple datasets produced by scanning each of several microarrays at multiple intensities. Our work was motivated by the hypothesis that the scan intensity required to achieve resolution necessary for detecting differential expression is inversely related to a gene's signal intensity. Thus, images containing low, intermediate and high numbers of saturated spots would be expected to detect differences in gene expression among genes that exhibit high-, medium- and low-signal strengths, respectively. To test this hypothesis empirically, three datasets, spanning a 20-fold difference in average signal strength, were analyzed to identify the number of statistically significant differences detected and the degree of overlap among the three datasets. Additionally, the multiple-scan images were analyzed using the linear regression (LR) procedure described by Dudley *et al*. (2002). Analysis of the multiple-scan result set supports our hypothesis and demonstrates that scanning at multiple intensities can play an important role in acquiring data for microarray analyses. Furthermore, the comparison between the datasets of the multiple-scan and the linear regression approaches demonstrates that the multiple-scan method identifies statistically significant differences in gene expression that the linear regression is unable to identify.

METHODS

Plant materials and anther collection

Anthers from maize plants of the inbred line Ky21 were collected at six distinct developmental stages and from two floret types (upper and lower).

RNA isolation and amplification

RNA was extracted from anthers using Trizol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's recommendations. Equal amounts of RNA from one to four individuals per stage were pooled randomly to generate one biological replicate. In total, 24 biological replicates (two biological replicates per stage per floret type) were generated. Approximately 100 ng of total RNA from each biological replicate were used as starting material for T7-based linear RNA amplification, performed as described by Nakazono *et al*. (2003).

Microarray procedures

Microarray protocols are available at http://schnablelab.plantgenomics.iastate.edu/resources/protocols/. Fluorescently-labeled cDNAs were prepared according to Nakazono *et al*. (2003) with slight modifications. Only targets that contained >3000 pmol of cDNA, >60 pmol of Cy dye and more than one dye molecule per 50 bases were hybridized to a 12 160 element cDNA microarray chip (Generation II version B) generated at Iowa State University's Center for Plant Genomics (http://www.plantgenomics.iastate.edu/maizechip/).

Microarray experimental design

For each of the two biological replications, upper and lower floret samples from each developmental stage were compared on two slides using a dye-swap design (Kerr *et al*., 2000). In addition, for each experimental replication, direct stage-to-stage comparisons were made within each floret type using a loop design (Kerr and Churchill, 2001). Hence, considering the two floret types, six stages and two biological replications, a total of 48 slides were used: 12 per replication for direct floret type comparisons and 12 per replication for stage-to-stage comparisons within floret types.

Microarray analyses

Arrays were scanned with a ScanArray 5000 (Packard, Meriden, CT). Initial scans were conducted at 50 μm resolution, and the laser power and PMT gain were adjusted until the ratio of the Cy3 and Cy5 channels was approximately one for a majority of the spots. A series of six scans, in ascending order of laser power and PMT gain, was then performed at 10 micron resolution and 50% scan rate. Initial laser power and PMT gain were ~78 and 70 for Cy3, and 75 and 57 for Cy5, respectively. For each successive scan, the laser power and PMT gain were increased by 3–4 units and 2–3 units, respectively. Fluorescent signal intensities were determined using ImaGene 5.0 (Biodiscovery, Marina Del Rey, CA). For each slide, dye and scan intensity, the median of the un-normalized log median signal intensities of all spots was computed using the R project for statistical computing (http://www.r-project.org/). For each slide and dye, the three scans whose medians were closest to 6.0, 7.5 and 9.0 were selected for analysis.

Linear regression of data at multiple scan settings

For each channel on two given scans, the linear regression algorithm (Dudley *et al*., 2002) was applied to the background-corrected spot intensities between the low and high detection limits at the two settings. The signal intensities of saturated spots were iteratively and linearly extrapolated using the unsaturated data at the low laser power and PMT setting.

Data normalization

An R implementation of the *lowess* normalization method (Dudoit and Fridlyand, 2002) was used to normalize the two channels for each combination of slide and scan intensity. The *lowess* normalization procedure was applied to the natural log of the background-corrected median signal intensities (median signal intensity minus the median background intensity) computed for each spot. The average of these *lowess*-normalized values across spots was computed for each slide, channel and scan intensity. The average of these averages was 5.7, 7.2 and 8.7 for the low, medium and high scans, respectively. The *lowess*-normalized data for each channel were then centered on the average for its scan intensity so that all channels sharing a common scan intensity would have identical averages. We refer to these *lowess*-normalized and mean-centered values as normalized signal intensities.

Statistical analysis

For each scan, a mixed linear model analysis was conducted separately for each of 12 160 spots using a strategy similar to that of Wolfinger *et al*. (2001). The mixed linear model included fixed effects for developmental stage (six levels), floret type (two levels), stage-by-floret interaction and dye (Cy3 or Cy5). Replication, stage-by-replication, stage-by-floret-by-replication, slide nested within replication and an observation-specific error term were included as random effects. These random effects were selected to allow for correlations among observations expected to result from the structure of the experimental design. A variety of tests were conducted as part of the analysis of the data. Only the test for expression change across stages is considered here for ease of exposition. Post-statistical analysis, 1245 spots were excluded from downstream analysis because the cDNA inserts from the corresponding EST clones had failed quality tests during the PCR probe generation step.

Quantitative real-time PCR

Gene selection and primer design Seventeen ESTs that exhibited significantly different expression for stage comparisons in at least one of the scan settings in the microarray experiments and fold changes of ~2-fold or greater were selected for expression validation via quantitative RT–PCR (qRT–PCR). Primers were designed such that predicted melting temperatures

were between 58 and 61°C, lengths were between 18 and 24 bases, the guanine–cytosine content ranged from 40 to 60% and predicted amplicon lengths were between 80 and 200 bp. The specificity of each primer pair was confirmed by BLAST analyses against the MAGI (http://magi.plantgenomics.iastate.edu/) and GenBank databases.

Reverse transcription

Two stage-specific aRNA replicates were generated for each stage (excluding stage 3) by pooling equal amounts of aRNA from the upper and lower floret samples. Each 800 ng aRNA pool was spiked with 1 ng of *in vitro* transcribed RNA from a human gene (GenBank accession no. AA418251) and used as template for reverse transcription as described by Nakazono *et al*. (2003), except that both oligo(dT) and random hexamers were used as primers.

Quantitative real-time PCR All 17 primer pairs yielded an apparently single amplicon (as determined by via agarose gel electrophoresis and dissociation curve analysis) and exhibited primer efficiencies with a correlation coefficient >0.99. These primer pairs were used for qRT–PCR analysis on an ABI GeneAmp 5700 sequencing detection system using SYBR Green I master mix (Applied Biosystems, Foster City, CA). Forty cycles of PCR were performed on each primer pair at an annealing temperature of 60°C, 200 nM each primer and 1 mM magnesium chloride with a 1:200 dilution of each cDNA pool (per biological replicate) as template; reactions were performed in triplicate. The *E*-value (i.e. 1 + PCR efficiency) was obtained from the dilution curve and the mean C_t values of each sample for all genes were calculated and used for fold-change calculations as described in Pfaffl (2001).

RESULTS

Selection of low-, medium- and high-intensity scans

Relative levels of transcript abundance in developing maize anthers were compared at six developmental stages using cDNA microarrays (Supplementary Figure 1). We hypothesized that images obtained using low-, intermediate- and high-scanning parameters would be expected to detect differences in gene expression among genes that exhibit high-, medium- and low-signal strengths, respectively. To test this hypothesis, each hybridized microarray was scanned six times in ascending order of laser power and PMT gain. Scans with median values of ~6.0, 7.5 and 9.0 for the natural log of the signal median intensity of the non-normalized data were classified as low-, medium- and high-intensity scans, respectively. Therefore, the low- and medium-intensity scans, and medium- and high-intensity scans differ by 4.5-fold signal strength, whereas the signal strengths of the low- to high-intensity scans differ by 20-fold. Figure 1 shows representative low-, medium- and high-intensity scan false-color images generated by the ScanArray software for a single region of one array.

Statistical analysis

Separate, equivalent statistical analyses were performed on the low-, medium- and high-intensity scan normalized datasets using a mixed linear model similar to that described by Wolfinger *et al*. (2001). As part of the mixed linear model analyses performed for each scan, tests for stage main effects were conducted for each of the 12 160 spots. The tests for stage main effects identify genes whose expression differed significantly across developmental stages.

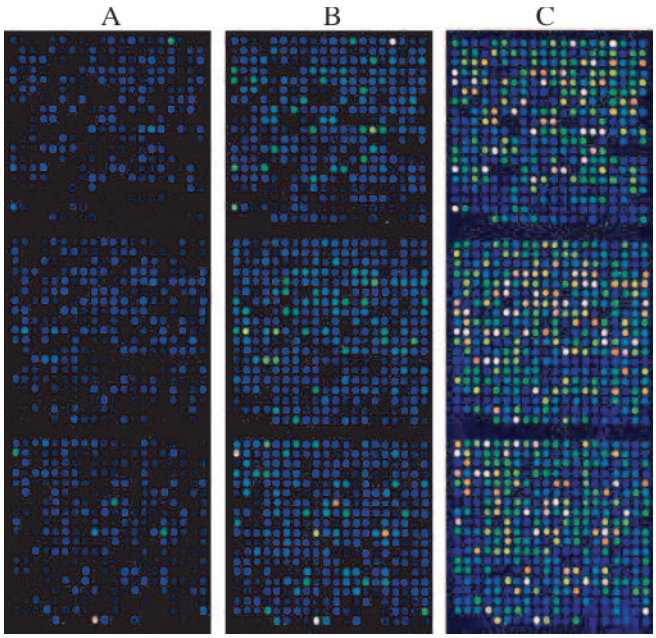

Fig. 1. Representative examples of low, medium, and high-intensity scans. Images were acquired from the same array by scanning in ascending order of laser power and PMT gain to generate the low (**A**), medium (**B**), and high (**C**) intensity scans. False-color images generated by the ScanArray software are shown based on signal intensity. The false-color scale, in ascending order of signal strength, is black (no detectable signal), blue, green, yellow, orange, red, and white (saturation).

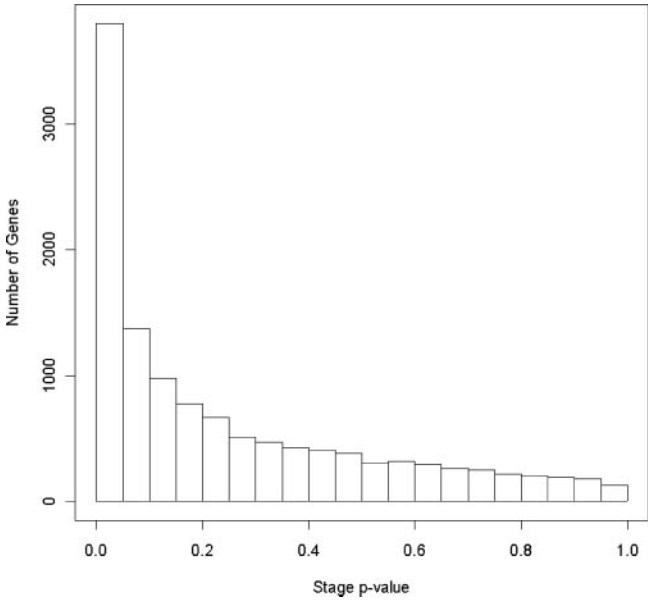

Fig. 2. Distribution of the stage *P*-values for the medium-intensity scan. The overabundance of genes with statistically significant differences for the small *P*-values indicates that many genes are differentially expressed across stages.

Identification of statistically significant differences

A histogram of *P*-values corresponding to the tests for stage main effects from the medium intensity scan dataset is depicted in

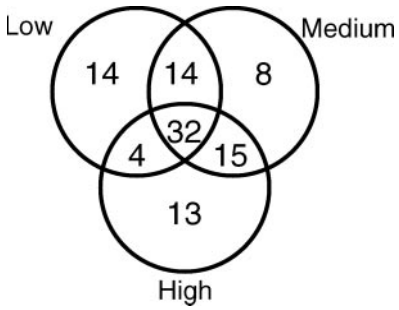

Fig. 3. Determination of the extent of overlaps among the low-, medium- and high-intensity scans. The three circles indicate the subsets of statistically significant differences identified by the low-, medium- and high-intensity scans for the stage comparisons. The numbers within the circles represent the percent of the non-redundant, statistically significant differences identified by each scan type. For the stage comparisons, a total of 398 non-redundant, statistically significant differences were identified at $P = 0.001$.

Figure 2. If no genes were differentially expressed across stages, the histogram would be expected to exhibit a uniform (i.e. flat) shape. Instead, there is a clear overabundance of small *P*-values, suggesting that many genes exhibited differential expression across stages. The analogous *P*-value histograms for the low- and high-intensity scans are highly similar (data not shown). The *P*-values for all 398 statistically significant genes at each of the scan intensities are provided in Supplementary Table 1.

Distribution of significant differences among the three scans

At the 0.001 *P*-value threshold for significance, the estimated false discovery rate was below 2% [as calculated using the method described by Storey and Tibshirani (2003), for each family of tests]. Using the 0.001 *P*-value threshold, a total of 398 non-redundant, statistically significant differences were detected in the stage comparisons after combining statistically significant differences from the low, medium and high datasets.

Comparisons among statistically significant genes from the low, medium and high datasets revealed that 32% were identified in all three scan intensities and each scan intensity identified 8–14% of unique spots (Fig. 3). Using a single-scan approach (i.e. low, medium or high) ~70% of the total non-redundant significantly different spots from the combined dataset were detected (Fig. 4). By combining two of the scan intensities, 86–92% of the total non-redundant significantly different spots were identified, and the addition of a third scan resulted in an 8–14% increase in the number of statistically significant differences detected for the stage comparison (Fig. 4).

P-value comparisons between the low and high scans

Figure 5 shows a scatter plot of −log base 10 of the stage *P*-values from the high-intensity scan against −log base 10 of the stage *P*-values from the low-intensity scan. Points in the upper and lower right quadrants of the plot represent genes whose stage *P*-values were <0.001 for the low scan data, and points in the upper left and right quadrants of the plot represent genes whose stage *P*-values were <0.001 for the high scan data. Although there is a strong correlation between the two sets of *P*-values, there are many genes for which the high- and low-intensity scans yield different conclusions.

Relationship between signal strength and identification of significant differences

An example of a gene (Gene #4318; GenBank accession no. DV492499) exhibiting statistically significant differences in the low-intensity scan but not in the high-intensity scan is presented in Figure 6. The two plots show the normalized log-scale signal intensities from the low- and high-intensity scans. The lines in each plot connect pairs of points obtained from a single slide. Solid and dashed lines represent slides from the first and second replications of the experiment, respectively. To improve clarity of the plots, data from slides corresponding to within-stage comparisons of floret types have been omitted from the plots. In the two plots, data from the low-intensity scan provide strong evidence of an increase in expression at stage 4 (stage *P*-value = 0.00038), while data from the high intensity scan provide little evidence of a statistically significant difference among stages (stage *P*-value = 0.33995). Interestingly, Gene #4318 has a high average signal intensity at each scan intensity; its within-scan average signal across all studied conditions exceeds the within-scan average signal of over 99% of the arrayed genes, regardless of the scan intensity considered.

The data for Gene #4318 depicted in Figure 6 illustrate a more general phenomenon: differential expression in high signal strength genes tends to be more readily detected with the low-intensity scan than with the high-intensity scan. To test whether this holds true more generally, the mean expression value for each differentially expressed gene (*P*-value $\leq$ 0.001) detected by each of the low-, high- and medium-intensity scans was plotted. In Figure 7 the mean signal of a gene refers to the average normalized log-scale signal of the gene over all three intensity scans and all conditions studied in the experiment (an average of $3 \times 96 = 288$ values). The distribution of mean signal values of detected genes decreases as the scan intensity increases. This indicates that the significantly different genes identified by the low-intensity scan tend to have a higher signal strength than significantly different genes identified by the medium-intensity scan, and that the significantly different genes identified by the medium-intensity scan tend to have higher signal strength than significantly different genes identified by the high-intensity scan.

Only significantly different genes with *P*-values $\leq$0.001 were selected in Figure 7. However, the trend depicted in Figure 7 persists for a variety of other criteria for differential expression, including *P*-value thresholds ranging from 0.0001 to 0.05 and *q*-value thresholds (Storey and Tibshirani, 2003) ranging from 0.01 to 0.05 (data not shown).

Figure 8 shows the relationship between the mean signal across all experimental conditions in the high- and low-intensity scans for all of the genes on the microarray. The vertical trend in the lower left corner of the plot indicates that the high intensity scan detected much more variation among the genes with the lowest signal strength. The horizontal trend in the upper right corner of the plot indicates that the low scan detected greater variation among the genes with the highest signal strength. This latter trend is primarily due to saturation of spots associated with high-signal genes scanned at high intensities. The plot is consistent with the idea that low intensity scans will provide better resolution of detecting

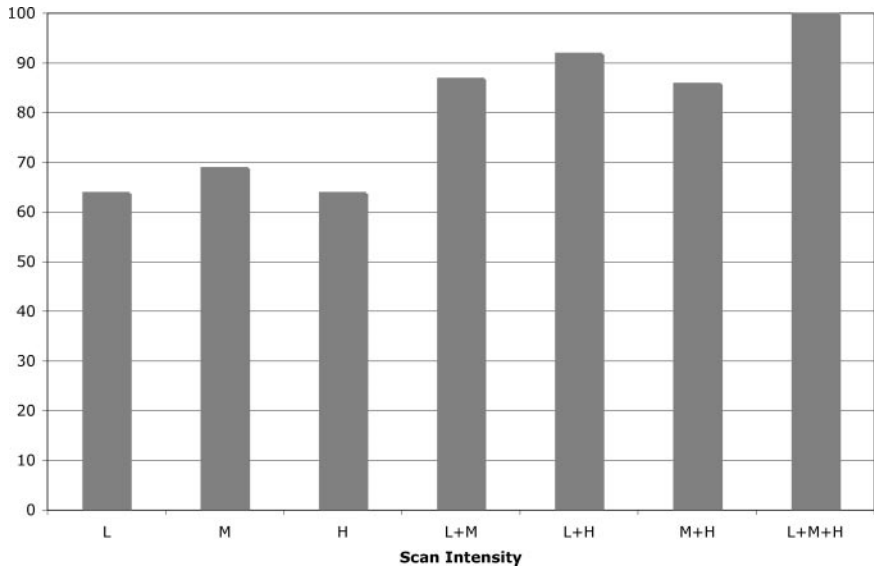

Fig. 4. The number of statistically significant differences that can be detected increases when the datasets from multiple scan intensities are combined. Scan intensities are: L = Low, M = Medium, H = High, L + M = Low and Medium, L + H = Low and High, M + H = Medium and High, L + M + H = Low, Medium and High.

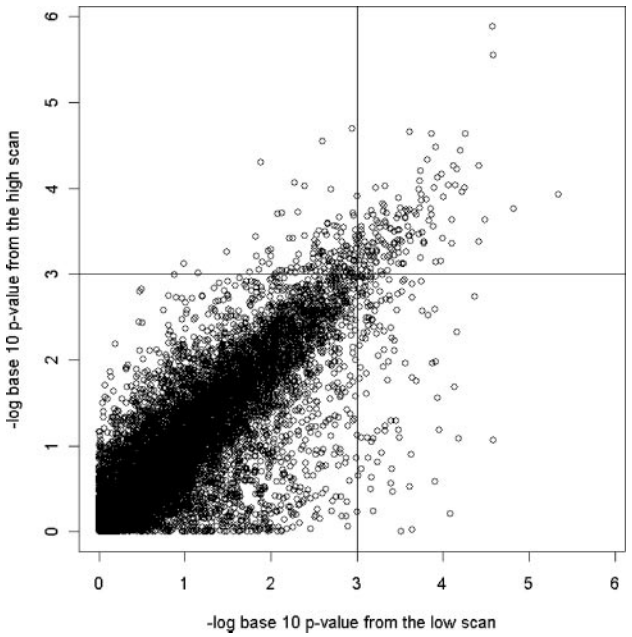

Fig. 5. Comparison of the stage *P*-values from the high- and low-intensity scans. Points represent individual spots. Points in the upper right quadrant are statistically significant ($P \leq 0.001$) for both the low- and high-intensity scans, points in the lower left quadrant are not statistically significant for either scan, points in the upper left quadrant are statistically significant for the high-intensity scan but not the low-intensity scan, and points in the lower right quadrant are statistically significant for the low-intensity scan but not the high-intensity scan.

differential expression among high signal genes while high intensity scans will provide better resolution for detecting differential expression for genes with lower signal levels.

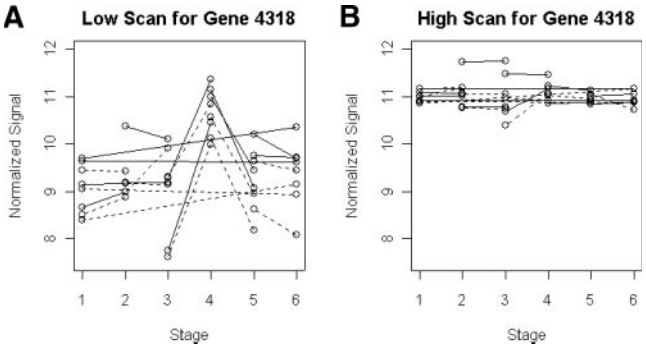

Fig. 6. Detailed examination of the signal intensity of a gene at low and high scan intensities. Gene #4318 was statistically significant for the low (**A**), but not the high (**B**), intensity scan in the stage comparison. Normalized expression for the statistically significant genes is plotted against stage.

Comparison of multiple-scan and linear regression methods

The linear range of signal for saturated spots can be extended by applying a linear regression model (Dudley *et al*., 2002). This algorithm corrects saturated signals by extrapolating the signal strength from low intensity scans. This algorithm was applied to the low, medium and high datasets (LR-LMH) to compare the resulting datasets with the multiple-scan method. The LR-LMH dataset yielded 291 statistically significant differences.

Investigation of the overlap between the *P*-values from the multiple scan and LR-LHM analyses is shown in Figure 9. Overall, there is a strong correlation between the two sets of *P*-values. However, the multiple scan approach is able to identify statistically significant differences ($P < 0.001$; Fig. 9, quadrants II and III) that were not detected by the LR approach. Conversely, the LR approach identified very few statistically significant differences ($P < 0.001$)

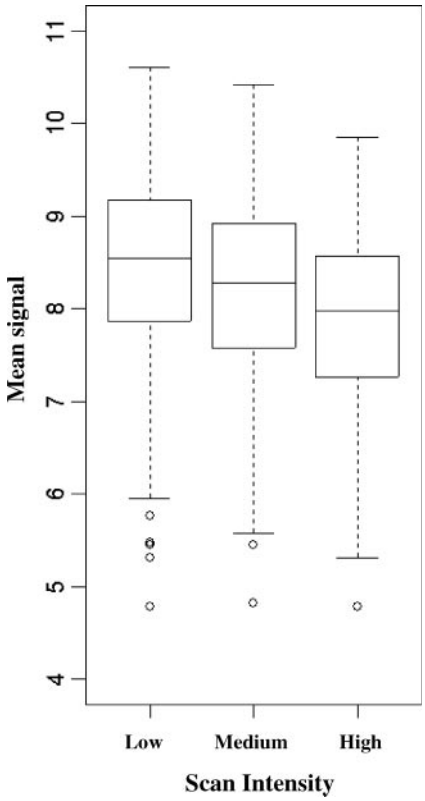

Fig. 7. Mean signal distribution for genes with P-values ≤ 0.001 for the low-, medium- and high-intensity scans for the stage comparisons. Mean signal was calculated by averaging expression values of each statistically significant gene over all three intensity scans and all conditions studied in the experiment (an average of $3 \times 96 = 288$ values).

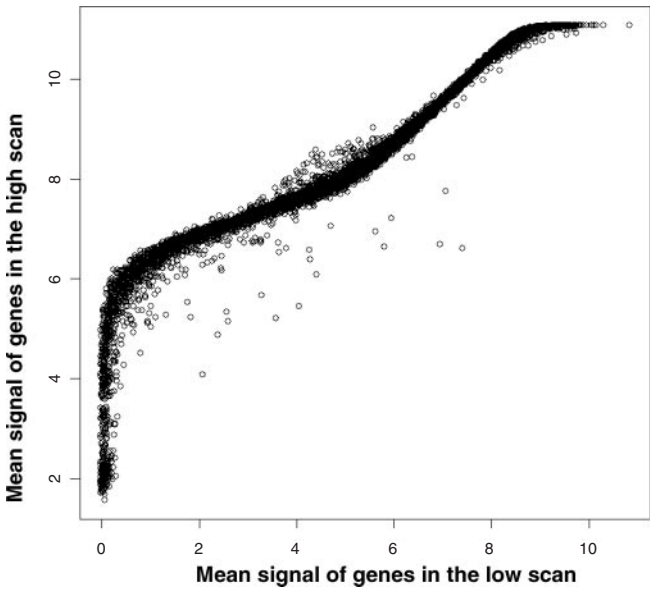

Fig. 8. A comparison of the mean signal for all genes on the microarray between the high- and low- intensity scans across all experimental conditions. The mean signal levels are lowest at the intersection and highest at the right of the x-axis and top of the y-axis.

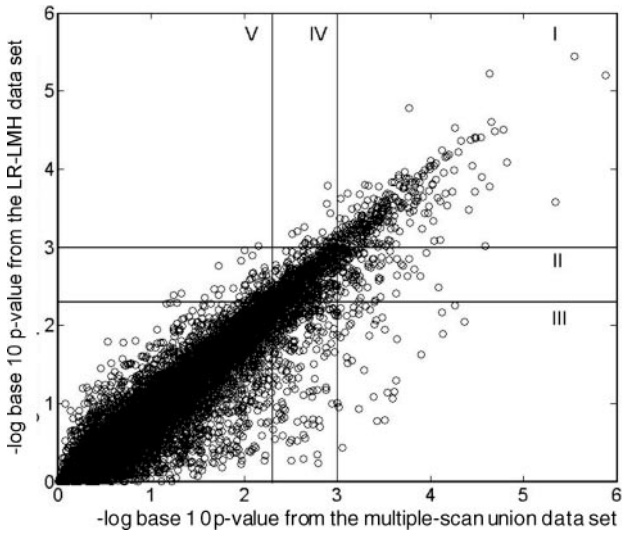

Fig. 9. A comparison of the P-values from the LR-LMH and the multiple-scan union datasets at two P-value thresholds. For each spot (represented as an open circle), the P-value of the multiple-scan union is the minimum P-value among the three datasets. Quadrant I contains spots with $P \leq 0.001$ for both the multiple-scan union and the LR-LMH datasets; Quadrant II contains spots with $P \leq 0.001$ for the multiple-scan union and $0.001 \leq P \leq 0.005$ for the LR-LMH dataset; Quadrant III contains spots with $P \leq 0.001$ for the multiple-scan union and $P \geq 0.005$ for the LR-LMH dataset; Quadrant IV contains spots with $P \leq 0.001$ for the LR-LMH and $0.001 \leq P \leq 0.005$ for the multiple-scan union; Quadrant V contains spots with $P \leq 0.001$ for the LR-LMH and $P \geq 0.005$ for the multiple-scan union.

that the multiple scan approach failed to identify (Fig. 9, quadrants IV and V).

Validation of microarray results using qRT–PCR

Supplementary Figure 2 shows a plot of the $\log_2$ fold change estimated from qRT–PCR versus the $\log_2$ fold change estimated from our microarray experiment. The correlation between the estimates was 0.773. There were two outlying points where the qRT–PCR and microarray estimates differed substantially. Without these points, the correlation was 0.899. Both correlations were statistically significant at well below the 0.001 level. For one of the outlying points (spot 768), the direction of the fold change according qRT–PCR was completely reversed from that estimated by microarray. In the other case (spot 8911), the qRT–PCR and microarray experiments both suggested a large fold change in the same direction, but the qRT–PCR results were more extreme than those obtained from microarrays. The direction of fold change estimated by microarray was the same as that estimated by qRT–PCR for 16 of the 17 genes. The fold change estimated from the microarray experiment along with the fold change estimates for each biological replication of the qRT–PCR experiment are provided for all 17 genes in Supplementary Table 2.

DISCUSSION

Because DNA microarray experiments are multi-faceted and complex, researchers are faced with many decisions, including determining the most efficient method to extract meaningful

data. One often-overlooked aspect of microarray experiments is data acquisition. Most protocols recommend scanning at laser settings that decrease the number of saturated spots. While this approach captures a subset of the statistically significant differences, it potentially excludes genes on the basis of signal intensity. By conducting separate analyses of data obtained using different scan intensities, we have demonstrated that no single scan intensity is optimal for all genes. Each scan intensity identified a unique set of statistically significant differences in gene expression; only approximately one-third of the statistically significant differences were detected in all the three scan intensities (Fig. 3). By combining the statistically significant datasets of three of the scan intensities, 30–40% and 10–15% more statistically significant differences are detected than single scan intensity and double scan intensity approaches (Fig. 3). Furthermore, the low intensity scan tended to identify high signal strength genes, whereas the high intensity scan tended to identify the low signal strength genes (Figs 7 and 8). One unexpected finding was that genes with low signal intensity were also identified (and validated) from the low intensity scan. This result could be explained by the high signal-to-noise ratio at the low scan settings (data not shown).

Signal detection for low signal strength spots is a limiting factor for the detection of statistically significant differences in microarray experiments (Duggan *et al*., 1999). Furthermore, low signal strength spots often exhibit pixilation, which contributes to variability (Romualdi *et al*., 2003). These low signal strength spots would be expected to be prevalent in the low intensity scans (Fig. 1a). However, as scan intensity increases, the proportion of low intensity spots decreases (Fig. 1b and c). The observation that the mean signal of the significantly different spots is inversely related to the scan intensity (Fig. 7) supports the conclusion that high intensity scans can increase the power for detecting expression differences in low signal genes.

Conversely, some spots on the array exhibit high signal intensity relative to the majority of the spots. When the signal from these spots becomes saturated for each channel, differences in transcript abundance cannot be detected. Therefore, to identify differences in gene expression for these spots, it is important to lower the laser settings until the spots are no longer saturated.

If pronounced, photobleaching (Nagl *et al*., 2005) could affect our multiple scanning strategy. To directly test the degree of photo bleaching under our conditions, a slide hybridized with Cy3 and Cy5 was scanned 15 times for each channel. In the Cy5 channel after an initial drop (∼25%) in the median intensity the remaining scan medians dropped only ∼4% from one scan to the next. The Cy3 channel also exhibited an initial drop (∼20%) in median intensity but over the next four scans exhibited an increase in median intensity before leveling off for the remainder of the scans. Hence, and in agreement with the results of Romualdi *et al*. (2003), we conclude that the effects of photobleaching are modest, at least when using the protocols described here.

Recommendations for researchers using cDNA microarrays

Although scanning is often overlooked as an important factor for microarray experiments, the results presented here demonstrate that scanning parameters can substantially affect the dataset generated. Unfortunately, many authors fail to report scanning parameters. We, therefore, recommend that authors report their scanning parameters.

Scanning at multiple intensities is a cost-effective method for extracting additional information at a minimal cost. The most effective method for maximizing the signal-to-noise ratio across multiple scan settings is to increase the laser power and keep the PMT gain constant (X. Zhao and L.A. Borsuk, unpublished data). We, therefore, encourage researchers using microarrays to scan at multiple intensities because no one scan intensity can be expected to maximize the power to detect differential expression for genes of varying signal strength. Although the scan intensity levels and analyses described here were useful for identifying differential expression in genes of varying signal strength, development of an optimal scanning and data analysis strategy remains an area for further investigation.

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