DNA Millichips as a Low-Cost Platform for Gene Expression Analysis\textsuperscript{[W][OA]}

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Our goal was to create a DNA chip that is as easy, convenient, and inexpensive as an agarose gel. For a first-generation solution, we describe a low-cost, easy-to-use de novo synthesis oligonucleotide microarray technology that draws on the inherent flexibility of the maskless array synthesizer for in situ synthesis of thousands of photolithographically produced oligonucleotides covalently attached to a microscope slide. The method involves physically subdividing the slide into $1 \times 1$ mm millichips that are hybridized to fluorescent RNA or DNA of biological origin, in a microfuge tube at an ordinary laboratory benchtop, rather than in dedicated hybridization chambers. Fluorescence intensity is then measured with a standard microscope rather than sophisticated DNA chip scanners. For proof of principle, we measured changes in the transcriptome of Arabidopsis (Arabidopsis thaliana) plants induced by growth in the presence of three major environmental abiotic stresses (temperature, light, and water status), in all possible combinations. Validation by comparison with quantitative reverse transcription PCR showed a high correlation coefficient and analysis of variance indicated a high technical reproducibility. These experiments demonstrate that low-cost DNA millichips can be made and reliably used at the benchtop in a normal laboratory setting, without assistance of core facilities containing costly specialized instrumentation.

Over the past decade, genomic research has seen an explosion in the quantity of data being produced from high-density microarrays and next-generation DNA sequencing. These technologies have been critical to an accelerating pace of knowledge acquisition and innovation. Although the use of increasingly larger data sets is crucial for elucidating the complexity of biological pathways, the generation, manipulation, and evaluation of these data require specialized equipment (e.g. hybridization chambers, scanners) along with advanced computer programming and statistical analysis skills typically found only in dedicated core facilities or groups dedicated to this type of research. Furthermore, the costs associated with these techniques can place them outside the reach of a typical bench scientist on a day-to-day basis.

The goal was to devise a method that would make DNA chips as accessible, inexpensive, and convenient for individual laboratories to use as agarose gels. For this purpose the maskless array synthesizer (MAS) was used to create DNA chips with a high density of long, single-stranded DNA sequences. The MAS is an automated instrument that utilizes the Texas Instruments digital micromirror device (DMD; Sampsell, 1994) to perform photolithography and is uniquely capable of de novo synthesis of long (e.g. 60-mer) oligonucleotides on a glass surface with a density that exceeds 1 million or more probes patterned within a 2-cm squared glass surface and affords the capability of programming any desired probe sequence of reasonable length (Singh-Gasson et al., 1999; Cerrina et al., 2002; Nuwaysir et al., 2002).

Because the cost of reagents for an entire/whole MAS-derived DNA chip on a microscope slide is around $450 it was reasoned that if the surface of a full-scale microarray slide was subdivided into many smaller but reproducible pieces, one could obtain a chip with a few thousand custom probe sequences at a price rivaling that of an agarose gel (i.e. in the order of tens of dollars rather than hundreds of dollars). Furthermore, if standard laboratory equipment could be used for processing the millichips (e.g. thermal cycler versus custom hybridization chambers, fluorescent microscope versus scanner, etc.) this would eliminate the need to purchase specialized equipment. Together these changes increase the accessibility of microarray technology for routine, daily usage in a standard molecular biology laboratory setting.

RESULTS

The Millichip Design

A critical goal of this study was to include enough probes on each millichip to make the resulting experiments scientifically valuable yet provide enough physical
separation of the chip segments to allow adequate millichip yield from a standard glass microarray slide. As a first step, a 1 × 1 mm millichip was found to meet these criteria (Fig. 1). A millichip of this size was accommodated by standard PCR tubes (e.g. 0.2 mL), was easily held with a reverse-acting tweezers, and could contain a 67 × 67 matrix of probe spots (i.e. 4,489 probe spots). From a yield perspective, it was determined that the best method for separating the millichip from the slide was to (1) score the slides prior to probe synthesis on the MAS; (2) align the scored slides to the MAS DMD and perform the probe synthesis; and (3) postsynthesis, manually separate the individual millichips from the slide by depressing a fine-tipped metal stylus against the slide surface opposite to the scoring (i.e. the probe surface of the slide). Initial yields of usable millichips from this manual process ranged from 50% to 70%. Although not explored as part of this study, process improvements (e.g. optimizing the prescoring depth and cut shape) along with automation could undoubtedly improve this yield.

A method was sought to eliminate the need for specialized equipment, such as hybridization chambers and scanners, to hybridize and measure the fluorescent intensity of the DNA millichips. It was determined that the 1 × 1 mm millichip could be conveniently placed in the bottom of a 0.2-mL PCR tube for hybridization of fluorescently tagged DNA or RNA targets to the complementary sequence synthesized on the glass surface. Various reaction volumes and conditions were explored with emphasis placed on minimizing volume to keep reagent expense low. As little as 10 μL of hybridization solution adequately covered the entire millichip and provided sufficient volume for a successful hybridization. To wash nongeneric label off the glass surface, a reverse-acting tweezers was used to hold the millichips securely and to allow their transfer between wash solutions contained in 250-mL standard glass laboratory beakers. This procedure minimized the risk of marks and other damage to the surface that would negatively impact optical measurements of fluorescent intensity.

To provide a routine and inexpensive method for measuring fluorescent intensity and data extraction, the use of a standard fluorescent microscope rather than a dedicated (i.e. expensive) scanner, as is currently used for DNA chips, was explored. By operating at 100× magnification with the fluorescent microscope, it is possible to capture the fluorescent intensity of each millichip with four overlapping 16-bit TIFF images. Software was written to merge data from the four images into a single dataset and fluorescent measurements could be quantified and dropped into a spreadsheet file for evaluation by the user. This program is freely available for download at http://www.biotech.wisc.edu/sussmanlab/Downloads. This stand-alone software operates with Microsoft Windows (i.e. requires no other software to run) and performs the following functions: accommodates images from a standard fluorescent microscope (e.g. 16-bit TIFF images); adjusts the angle, size, and position of an image; automatically adjusts the spacing between spots to compensate for stretched images; gives the user the option of excluding damaged or contaminated areas of the millichip from further evaluation (Fig. 2); and manipulates the raw pixel data to calculate minimum, maximum, average, median, and SD as well as background correct probe data. This study employed a method of background subtraction that utilizes blank spots adjacent to probe spots, but additional approaches are currently being incorporated. The experiments described here looked only for large changes in expression levels, allowing the use of simpler techniques. In future work, mismatch probes and other standard techniques will be incorporated into the array design to allow for more advanced background correction and normalization methods. This system of measuring and recording fluorescent intensity with a microscope found in routine use in most biology laboratories enables an untrained user to evaluate the

Figure 1. Millichip process. A, Standard glass microarray slides are prescored to a depth of 0.7 mm prior to synthesis on the MAS. B, The glass slide is broken down into millichips along the prescored grid. C, Hybridization is carried out in 0.2-mL PCR tubes with as little as 10 μL solution. D, Millichips are held with reverse-acting tweezers for the washing steps after hybridization. E, Each millichip is imaged using a standard fluorescence microscope. The image shown is the array of a single millichip.
transcriptome of an organism or tissue type without the need for specialized scanners.

Examining a Stress Response in Arabidopsis (Arabidopsis thaliana)

An experiment was designed to test the ability of millichips to provide biologically useful information and demonstrate the capability to readily produce and use a large number of individual chips in a single study. As the initial test of the millichip platform, we chose to examine several environmental perturbations under which plants have evolved that are related to the drought response: (1) temperature (heat, cold, or normal), (2) water status (dehydration, salt, or normal), and (3) light intensity (normal or high). In addition, we separately examined the effect of the dormancy hormone (+)-abscisic acid (ABA) to provide a comparison treatment for evaluating the millichip performance with previously published research. The genes represented on the array were chosen based on previous stress response studies in which arrays were used to monitor expression changes for a single treatment (Kreps et al., 2002; Rossel et al., 2002; Seki et al., 2002a, 2002b; Kimura et al., 2003; Taji et al., 2004; Huang et al., 2008; Kant et al., 2008; Matsui et al., 2008; Wohlbach et al., 2008; Abdeen et al., 2010). By choosing genes that showed large changes in expression under each single treatment, we were able to ensure that a range of expression levels would be present on each millichip because genes that show large changes in expression under one treatment may show little or no change under another. One advantage of smaller, more cost-efficient arrays is the ability to look at a smaller set of genes under a larger set of conditions. In this study it was possible to investigate the response to not only single stress treatments, but also every double and triple combination of these stresses. Table I describes the individual conditions as well as the double and triple combinations of conditions that were tested in this study. There were a total of 18 treatments plus two controls (i.e. normal/untreated and ethanol treated for the ABA comparison) used for this initial study.

With the ability to produce hundreds of millichips, it is possible to look at combinations of treatments in a way that was previously inaccessible to many labs. The objective of this study was to demonstrate the use of the millichip as a quantitative tool to screen for large expression changes and not for the qualitative study of expression levels. To this end, simpler methods of normalization and analysis were used in the screening experiments with the ability to verify interesting and significant results by quantitative PCR, additional chip experiments, or by other methods. Thus, expression fold changes of less than two were categorized as no change.

Verification of the Millichip Platform

To determine statistical reliability, we performed an average of three to four technical replicates and six biological replicates for each treatment and control group, resulting in a total of 457 individual millichip experiments for this study.

For the initial investigation, a simplified millichip utilizing around 1,000 out of 4,489 available spots was designed to not only provide an easy method of background correction exploiting blank spots, but also for the ease of image analysis while the software extraction and normalization functions were in development.

All millichips were identical in design with 1,156 total probe spots consisting of 911 unique gene probes derived from genes chosen from stress response studies as described previously; 17 actin genes represented by 32 probes; probes for reference genes ubiquitin, GAPDH, and 5SRRNA; four probes matching the Affymetrix GeneChip Poly-A RNA control kit sequences used to monitor target labeling; and five probe sequences that were the reverse complements of labeled spike-in oligos used to verify concentration-dependent signal response on each millichip. The actin, reference gene, and Affymetrix GeneChip label control probes were all replicated five times on each millichip whereas the spike-in concentration oligo probes were replicated nine times. To provide a more
valuable comparison, the majority of probes were designed based on commercially available arrays from Roche Nimblegen (Thibaud-Nissen et al., 2006), whereas an additional 67 probes were designed using the OligoArray software (Rouillard et al., 2003). These 911 gene probes did not have any replicates on individual millichip (i.e. replication of the 911 unique gene probes was a function of the number of technical replicates performed for each biological replicate).

The custom software previously described was used to extract the median pixel value for each probe and blank (i.e. empty/nonprobe) position in the millichip microarray matrix. An average value was then calculated for all the blank spots immediately adjacent to a probe position. The background-adjusted probe value was calculated by subtracting the average blank value from the probe value. This method of background adjustment to the probe values not only corrected for the higher background noise associated with using a standard fluorescent microscope versus a scanner but also corrected for fluorescent lamp uniformity variance inherent with the microscope’s arc lamp source. Background-adjusted probe values were converted to their log base-2 values, which were then normalized using the ratio of the probe distribution medians. Supplemental Table S1 provides a statistical summary for each probe under each treatment condition including treated and untreated normalized, absolute probe values, log2 ratio, fold change, and P value. The entire data set is available from the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE37118).

Figure 3A illustrates the concentration-dependent signal response for four representative millichip experiments, which as expected depicts increasing signal with increasing spike-in oligo concentration. This concentration-dependent signal response was checked for each millichip. The median coefficient of variation for the probes replicated on each individual millichip (i.e. actin, GAPDH, ubiquitin, and 5SRRNA) was 12% as shown in Figure 3B.

Histograms for the technical replicate signal intensity coefficient of variation (i.e. signal SD divided by signal average) show a 27% average coefficient of variation that while not quite as good as that obtained using traditional DNA chips is reasonable when one considers the low cost and ease of use (Fig. 4A). The average biological replicate signal intensity coefficient of variation of 26% observed in this experiment is also well within the range (approximately 20%) generally seen with whole-plant studies (Fig. 4B). Quantitative real-time PCR was used to validate fold changes derived from the millichip experiments (Fig. 5). A strong correlation between the two sets of data were observed, comparing favorably to previously published evaluations of normal high-density microarrays (Nuwaysir et al., 2002). Overall these observations demonstrate that in its initial formulation, the millichip platform performs well for the identification of genes that show a 2-fold or greater change in expression in a much larger number of treatments than is currently affordable or possible with resources found in a traditional molecular biology laboratory.

When possible, results were compared with previously published array data for other stress response studies in Arabidopsis to look for agreement in up-regulated and down-regulated genes. For the purpose of this study, fold changes less than 2-fold were considered to be no change. When comparing the data for cold, drought, and ABA treatment, agreement with

| Treatment | High Light | Heat | Cold | Salt | Dehydration | ABA |
|-----------|------------|------|------|------|-------------|-----|
| High light | x          |      |      |      |             |     |
| Heat      |            | x    |      |      |             |     |
| Cold      |            |      | x    |      |             |     |
| Salt      |            |      | x    |      |             |     |
| Dehydration |            |      |      | x    |             |     |
| ABA       |            |      |      |      | x           |     |
| High-light-heat | x | x    |      |      |             |     |
| High-light-heat-salt | x | x    | x    |      |             |     |
| High-light-heat-dehydration | x | x    | x    | x    |             |     |
| High-light-cold | x |      | x    |      |             |     |
| High-light-cold-salt | x | x    | x    |      |             |     |
| High-light-cold-dehydration | x | x    | x    | x    |             |     |
| High-light-dehydration | x |      | x    |      |             |     |
| Heat-salt | x          | x    |      |      |             |     |
| Heat-dehydration | x | x    |      |      |             |     |
| Cold-salt |            | x    | x    |      |             |     |
| Cold-dehydration | x | x    |      |      |             |     |
Previously published data were approximately 70%. Discrepancies in these types of comparisons arise from differences in experimental conditions such as treatment times and methods as well as the problems inherent in making comparisons between different array platforms.

**Data Clustering**

One objective of the biological experiment performed in this study was to investigate whether combining different environmental perturbations resulted in gene expression changes that were not predictable based on a study of those treatments performed individually. We believe this highlights an important use of the millichip platform. The ability to do hundreds or even thousands of unique chip experiments makes it possible to study more perturbations to a biological system. To examine this question, hierarchical clustering was performed (TIBCO Spotfire) with the normalized dataset (i.e. fold changes in gene expression are described by the ratio transcript signal intensity from treated plant tissue relative to untreated control plant tissue) by complete linkage with Euclidean similarity measure (Fig. 6; Kaushal and Naeve, 2004). This analysis of the mRNA changes observed in the various single, double, and triple treatments showed several relationships that were expected based on prior knowledge of the underlying biology. There were many genes on the chip that were positive controls based on known effects in the literature. For example, we observed that an ABA-responsive protein-related gene (AT5G52300.1) is up-regulated 80-fold in the ABA treatment, with relatively no change in the control treatment. Also noted was the heat shock protein-related gene (AT2G29500.1) that was up-regulated in the heat treatment, as well as all combination treatments that included heat. Additional examples of genes or groups of genes behaving as noted in prior literature are described in more detail in Supplemental Table S2.

In general, there were two major clades observed: clade I representing high-light-salt (LS), salt (S), cold-dehydration (CD), cold (C), cold-salt (CS), high-light-cold-salt (LCS), high-light-cold-dehydration (LCD), high-light-cold (LC), dehydration (D), high-light-dehydration (LD), and ABA; and clade II representing heat (H), heat-dehydration (HD), high-light-heat-dehydration (LHD), heat-salt (HS), high-light-heat-salt (LHS), and high-light-heat (LH), with heat being the dominant resolving feature creating the separation. Within clade I, it was also clear that cold caused a clustering separate from the other single treatments with clade Ia (cold-dehydration [CD], cold [C], cold-salt [CS], high-light-cold-salt [LCS], high-light-cold-dehydration [LCD], high-light-cold [LC]) sharing the cold variable. A dominant effect of temperature on gene expression, compared with the other treatments examined in this limited study can be explained by the realization that unlike warm-blooded animals that maintain a constant body temperature under many different external temperatures, the metabolism and growth of plant cells is strictly dependent on and greatly affected by the temperature of the environment. As known from many physiological studies, and the simple observation of annual tree rings from trees grown in environments with large seasonal swings in temperature, at low temperature plant cells stop dividing and the metabolism is greatly reduced. At the same time, at higher temperature, there is a nearly universal heat shock response in which gene expression is focused on producing proteins that offer protection against thermal denaturation. For example, three of the most highly up-regulated genes across the heat treatments code for heat shock proteins (AT2G29500, AT1G74310, and AT3G12580). The fold changes seen across the heat treatments for these genes range from 15- to 232-fold, relative to the control, in agreement with previous work (Kilian et al., 2007). It should be kept in mind that only 5% of the genes in the Arabidopsis genome are represented on this millichip.

Figure 3. A, Log2 concentration-dependent signal intensity for four typical millichips. B, Flourescent signal intensity coefficient of variation for actin, GAPDH, ubiquitin, and 5SRRNA probe replicates on each individual millichip.

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and they were chosen mainly to reflect those genes known or suspected to be involved in the plant drought response. The relationships observed from the cladogram may artificially reflect this bias rather than portraying underlying mechanisms in the organism’s response. In addition, although this hierarchical clustering appears to be the best fit given the parameters chosen for the analysis, there may be other equally plausible cluster results possible. As shown in Figure 5, there is no relationship between the number of genes that showed significant changes, and the position of that treatment within the cladogram (Table II). The single treatment with the fewest changes was the high-light treatment (24), followed by the cold-salt treatment (76), and the triple treatment of high-light-cold-salt (78). Salt treatment produced a much larger number (250) of genes that change significantly. Although salt is often considered a drought-inducing agent, the greater response to salt compared with drought (91) is not unexpected because in addition to acting as an osmolyte that pulls water out of cells, sodium chloride is permeant and the entry of large amounts of sodium and chloride ions in the cell is likely to cause a much greater change in the transcriptome than simple dehydration induced by air drying. Cold alone produced 97 changes whereas heat alone gave 250. As mentioned previously, from the number and pattern of genes induced by either low or high temperature, it is clear that the Arabidopsis plants are qualitatively more responsive to the temperature than any other single treatment.

**Combination Treatments**

Of particular interest is the question of whether there were genes that exhibited changes in the double and triple treatments that were not expected based on changes known to occur in single treatment conditions. Very few examples of combined treatment comparisons exist and, to the best of our knowledge, there are currently no examples of triple treatment comparisons, possibly due to the prohibitively high cost for the necessary number of arrays (Rizhsky et al., 2004). The millichip provides researchers with the ability to do these and similar experiments requiring hundreds or thousands of DNA chips cheaply and easily. There are several examples of unexpected results in expression in which the combined treatment shows expression in one direction and the corresponding single treatments show expression in the opposite direction. When the double treatment of high-light and cold (LC) is compared with the single high-light (L) and cold (C) treatments there are 160 genes up-regulated in both double and single treatments and 242 genes down-regulated in both double and single treatments (Supplemental Table S3). However, there are also 81 genes up-regulated in the high-light-cold treatment that are down-regulated in both the single high-light

![Figure 4.](image)  
**Figure 4.** Fluorescent signal intensity coefficient of variation for the normalized signal intensities for technical replicates (A) and biological replicates (B).

![Figure 5.](image)  
**Figure 5.** Log ratio comparisons of reverse transcription quantitative PCR and millichip microarray data for 32 different combinations of test conditions and gene probes. Correlation between the quantitative PCR and millichip data were 0.88 with a regression coefficient of 1.07.
and cold treatments and 23 genes that are down-regulated in the double treatment and up-regulated in both single treatments. More detailed information including gene identifiers and annotations is presented in Supplemental Table S4. The same comparison was made with triple treatments as related to double and single treatments and is summarized in Supplemental Table S5 with more detailed information in Supplemental Table S6.

Table II. Significant fold changes as compared with control

| Treatment                | <2   | >2  | Total |
|--------------------------|------|-----|-------|
| High-light-salt          | 112  | 159 | 271   |
| Salt                     | 120  | 91  | 211   |
| Cold-dehydration         | 69   | 46  | 115   |
| Cold                     | 63   | 34  | 97    |
| Cold-salt                | 46   | 30  | 76    |
| High-light-cold-salt     | 70   | 8   | 78    |
| High-light-cold-dehydration | 91  | 29  | 120   |
| High-light-cold          | 107  | 22  | 129   |
| High-light               | 15   | 9   | 24    |
| Dehydration              | 66   | 25  | 91    |
| High-light-dehydration   | 142  | 82  | 224   |
| (+)-ABA                  | 151  | 122 | 273   |
| Heat                     | 118  | 132 | 250   |
| Heat-dehydration         | 155  | 89  | 244   |
| Heat-salt                | 161  | 105 | 266   |
| High-light-heat-dehydration | 99  | 61  | 160   |
| High-light-heat-salt     | 173  | 76  | 249   |
| High-light-heat          | 171  | 89  | 260   |

In conclusion, it is clear that when environmental perturbations are grouped together in all possible double and triple combinations, a significant fraction of the genome sampled on this chip do not display changes in a direction predictable based on the single or even double treatments alone. Given (1) the size of the Arabidopsis genome (there are over 500 transcription factors known, out of the approximately 30,000 genes), (2) the complexity of the environment in which Arabidopsis evolved (light, temperature, nutrient starvation, gas concentration, to mention just a few), and (3) the large number of genes devoted to network cross-talk and other regulatory aspects of sensory perception and response (the largest single gene family in Arabidopsis is the protein kinase gene family, containing 1,000 members) it may not be surprising that the transcriptional apparatus does not respond in a simple fashion when the plant is perturbed.

DISCUSSION

Although DNA chips are still routinely used for DNA and RNA analyses, next-generation DNA sequencing technologies are also now becoming more commonly utilized for the same purposes. As DNA sequencing instrumentation offer higher throughput and multiplexing capabilities in next-generation experiments, they may provide opportunities for lower cost. However, ease of use and requirements for specialized instrumentation remain obstacles for efforts to make sequencing a viable alternative to the millichip technology described herein. In addition, it should be noted that DNA arrays are often utilized together with next-generation sequencing, for capture experiments.
that extend the utility and reduce the price of next-generation sequencing studies (Okou et al., 2007). Millichips should also be amenable for use in capture experiments, and may offer new low-cost and convenient opportunities for high-throughput studies that require the isolation of specific DNA or RNA sequences. Methods for millichip capture and other applications are currently under development.

We have shown that the millichip platform is a useful tool for screening genes that show a 2-fold or greater change in expression with a far greater number of biological variables possible. When it is desirable to identify genes with statistically significant changes less than 2-fold in magnitude, more traditional microarray approaches should be used. This technology shows great promise as a primary screen to examine gene expression in many separate treatments (e.g. 100 or more) than is currently possible. For most applications, the millichip flexibility and reliability combined with low cost and ease of use outweigh any limitations to detect minute changes.

Excluding labor, total material costs in this study were between $33 and $37 per millichip including all material costs associated with reverse transcription, amplification, and fluorescent labeling of the starting total RNA ($21 per millichip); synthesis of the milli ampli were between $33 and $37 per millichip including all low cost and ease of use outweigh any limitations to

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alone (95% ethanol). Heat-treated seedlings were incubated in a growth chamber maintained at 37°C, whereas cold-treated seedlings were incubated on ice in a growth chamber maintained at 10°C. For the salt treatment, seedlings were added to 10 mL of fresh MS media containing 200 mM NaCl.

RNA Isolation

Treated seedling tissue was blotted dry on tissue paper, weighed, flash frozen in liquid nitrogen, and stored at −80°C until RNA could be isolated. Total RNA was isolated from ground tissue using the RNeasy plant mini kit (Qiagen), including the on-column DNase digestion step.

Slide Preparation Prior to Microarray Synthesis

A series of cuts extending partially through the thickness of a standard glass microarray slide (Arrayyst) were machined at 1.5-mm intervals across the length and width of the slide surface (Mindrum Precision, Inc.). These cuts define the approximate 1 × 1 mm square millichip on the slide surface and served as break-lines during cleavage of the millichip from the slide after microarray synthesis. Cuts were machined approximately 75% through the slide thickness and created a grid pattern encompassing the millichip pattern projected by the MAS during microarray synthesis. A stock solution was prepared with 0.1% (v/v) glacial acetic acid in 95% (v/v) ethanol:water. At room temperature slides were immersed with rotation for 4 h in a solution consisting of 2% (v/v) N-(3-triethoxysilylpropyl)-4-hydroxybutramide (Gelest) in stock solution. Slides were then rinsed with rotation twice in fresh stock solution for 20 min. After a final diethyl ether rinse, slides were placed in a 120°C oven for 1 h followed by overnight curing under vacuum at 120°C (12–16 h). Functionalized slides were sealed and stored dry until used for synthesis (Beier and Hohbein, 1999).

Microarray Synthesis

Light-directed synthesis was performed using the MAS technology described previously (Singh-Gasson et al., 1999). Prescored slides were mounted in the standard slide holder used on the synthesizer and the cuts on the slide surface were aligned to the millichip images projected on to the slide through the use of a CCD camera (Watec) coupled to an infinity-corrected long working distance 20× M Plan APO objective (Mitotoyo) via a standard Infinity Photo-Optical mounted behind the transparent quartz reaction cell. Manual stages holding the reaction cell and slide assembly were used to position the cuts on the slide relative to the projected millichip image by observing their relative positions with the CCD camera assembly. Oligonucleotide probe synthesis was performed using DNA synthesis protocols previously described (Singh-Gasson et al., 1999; Cormina et al., 2002; Novayayar et al., 2002) where the removal of the photo-labile protecting group 2-nitrophenylpropyloxycarbonyl was performed by exposure to broadband UV-light wavelengths of g, h, and i lines produced by a 350 Hg arc lamp (Newport) and nucleotide base attachment achieved using standard phosphoramidite chemistry (Fodor et al., 1991). After completion of microarray synthesis, the slides were broken into individual millichips by pressing a small-diameter precision drill head eight-tube strips, and optically clear diethylpyrocarbonate-treated water using 1 unit of amplification grade DNase I (DNase I, Amp Grade, Invitrogen) followed by reverse transcription with the SuperScript III first-strand synthesis system for reverse transcription-PCR (Invitrogen). Real-time PCR reactions were performed with iQSYBR green supermix, clear 0.2-mL low-profile eight-tube strips, and optically flat eight-cap strips (Bio-Rad) in a Bio-Rad C1000 thermal cycler equipped with a Bio-Rad CFX96 real-time PCR detection system and CFX Manager software. Primers were designed with QuanPrime (Arvidsson et al., 2008) and purchased from a commercial vendor (IDT). All reactions were performed in accordance with the vendor-supplied protocols. Threshold cycle (Ct) values were extracted with the default threshold setting in the CFX Manager software and fold changes were calculated using the 2−ΔΔCt quantitative PCR data transformation (Schmittgen and Livak, 2008). The GAPDH gene was used as the reference gene.

Microarray data from this article can be found in the National Center for Biotechnology Information Gene Expression Omnibus data libraries under accession number GSE37118.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Stress response data set.

Supplemental Table S2. Data for genes with known function.

Supplemental Table S3. Comparison of double and single treatments.

Supplemental Table S4. Comparison of double and single treatments for specific genes.

Supplemental Table S5. Comparison of triple, double, and single treatments.

Supplemental Table S6. Comparison of triple, double, and single treatments for specific genes.

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