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REGULATORY RESEARCH PERSPECTIVES

Impact on Public Health

Technical Issues Involved in Obtaining Reliable Data from Microarray Experiments

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Abstract: Microarrays show great promise in advancing the understanding of many biological phenomena, including toxicity and effectiveness of the many products regulated by the U.S. Food and Drug Administration. In addition, devices based on the microarray technology have the potential to individualize diagnosis and treatment of disease, as well as monitor efficacy of treatment regimens. To realize these expectations, reliable and reproducible measurements are essential. Currently, there are no generally accepted standards for performing and analyzing a microarray study. Without such quality assurance standards among the microarray community, it will be difficult to move this technology into the regulatory framework where it holds such promise. Here, we describe observations and approaches undertaken at the NCTR Center for Functional Genomics to examine and optimize steps in the complex microarray procedure with the aim of decreasing variability in order to generate reliable data. These observations illustrate some of the subtle technical issues that can easily be overlooked in microarray experiments. Among the factors that can influence microarray experiments are microarray printing procedures, oligonucleotide characteristics, RNA quality, and environmental factors.

Introduction

The mission of the National Center for Toxicological Research (NCTR) is to conduct peer-reviewed scientific research that supports and anticipates the U.S. Food and Drug Administration's (FDA's) current and future regulatory needs (http://www.fda.gov/nctr/overview/mission.htm). This involves fundamental and applied research specifically designed to define biological mechanisms of action underlying the toxicity of products regulated by the FDA. It also includes the development of methods to improve assessment of human exposure, susceptibility, and risk. The Center for Functional Genomics at the NCTR has implemented DNA microarray technology that enables the evaluation of the effects of chemical toxicants on gene expression as well as the discovery of new biomarkers. To acquire high quality

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data that will permit definitive conclusions to be made about gene expression, it is important that all steps involved in this technology are optimized and standardized to reduce experimental error. Such need for standardization has been recently reviewed [1, 2]. The experiments reported here describe approaches undertaken to optimize each step and decrease variability in microarray technology in order to generate reliable data. In addition, an evaluation of automated microarray hybridization instruments is provided. Examples are given of spotted oligonucleotide glass slide microarrays, although many of the lessons learned are applicable to *in situ* synthesized microarrays, such as those from Affymetrix and Agilent Technologies [3-5], and cDNA microarrays [6, 7].

Microarray technology allows the relative gene expression levels to be determined among sets of biological samples, including tissue and cell samples from experimental animal models, humans, lower organism models, etc. In the field of toxicogenomics, which applies new high-throughput genomic technologies to toxicology, a typical application would involve the determination of gene expression changes associated with exposure to a toxic compound with the aim to understand mechanism or develop biomarkers of risk. A microarray experiment utilizes a complex multi-step process that is illustrated in **Figure 1**. Initially, a microarray of selected genes (called probes) is fabricated on a microscope slide. The genes in the experiments reported here are unique short oligonucleotides of approximately 50-80 nucleotides in length that are designed to have minimal homology with other genes. Collections of large numbers of genes (10,000 to 40,000) from various organisms, including the rat and mouse toxicology model systems, are available from commercial sources. These genes come in multi-well microplates with each gene in a well. The genes need to be dissolved in a printing buffer and often transferred to new daughter plates before the printing process starts. The transfer is too error-

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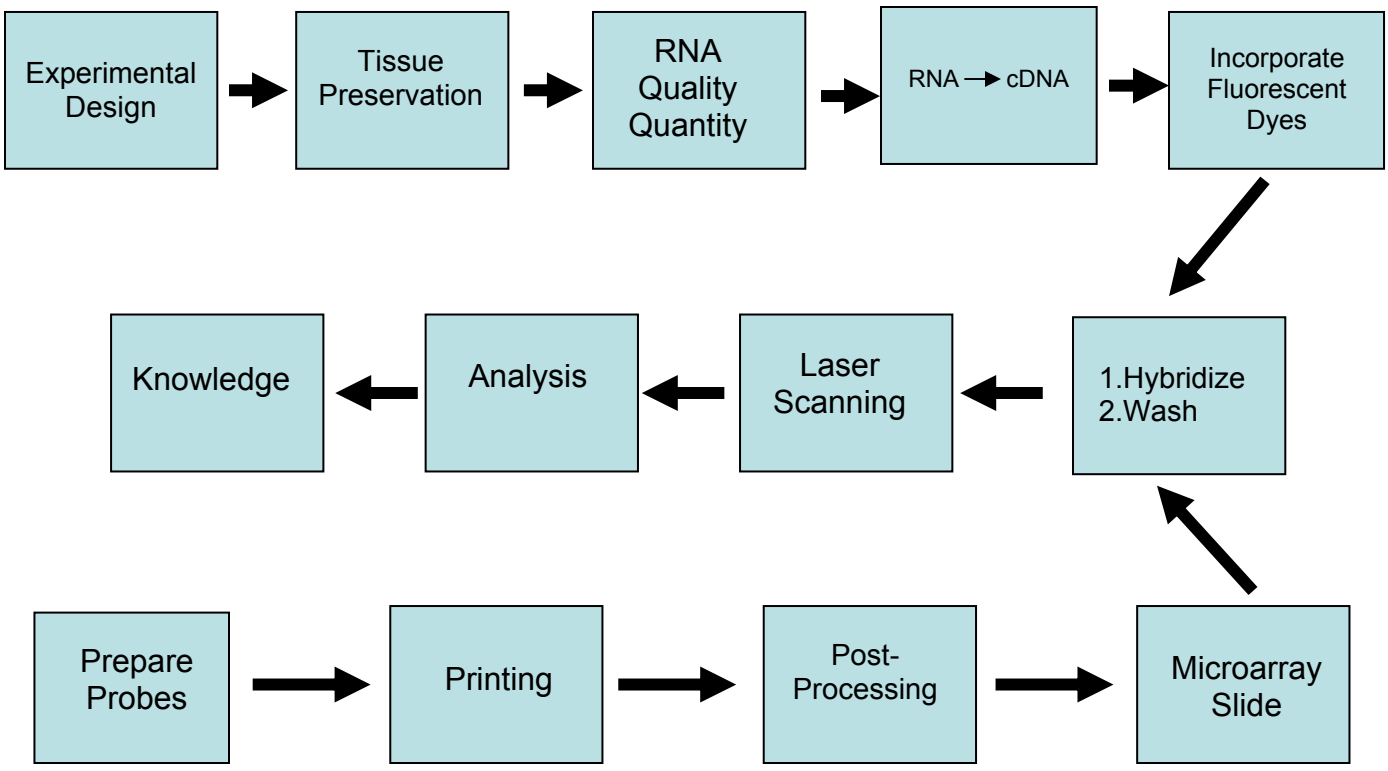

Figure 1. A microarray experiment. The top part of the figure shows steps in the sample preparation process while the bottom part shows steps in the fabrication of the microarray. The samples are hybridized to the microarrays (center). The net result of this complex multi-step procedure is increased understanding of the effects of chemical toxicants.

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prone for laboratory workers to perform manually and requires a high capacity, liquid-handling robot and sample-tracking software. The gene solutions are then deposited at known positions on the glass microscope slide and, after processing the slide to firmly attach the genes to the slide, to remove salt solutions, and to block reactive sites on the glass surfaces, the microarray is ready for use in an experiment.

The preparation of samples is also a multi-step process that starts with the collection of tissue or cells in a manner that preserves the RNA integrity. The RNAs are then used to create fluorescent targets that will bind by hybridization to their specific genes on the microarray. These targets are applied to the microarray slides under conditions that will allow specific and efficient binding. The targets that do not bind are subsequently washed away, leaving only the specifically bound targets. Using a high resolution fluorescence scanner, the fluorescent intensity at each gene position on the microarray is determined, and this value is used as a measure of expression of each gene. These data are then analyzed and used to develop knowledge about the effect of a particular drug, toxicant, or disease on the biological system. Suggested starting points have been published for those new to developing and using DNA microarray technology [8, 9]. However, optimizing all of these steps is a challenge, and there is ample opportunity for experimental variability to mask true biological effects. This manuscript discusses some of the main technical issues that must be considered and optimized to produce high quality microarray data.

A Gene Machines Default Pin Wash Procedure

1. Sonicate (2 sec.)
2. Loop (4 times)
   a. Wash (1 sec.)
   b. Dry (1 sec.)

B Modified Pin Wash Procedure

1. Wash (5 sec.)
2. Loop (4 times)
   a. Wash (2 sec.)
   b. Sonicate (2 sec.)
3. Wash (4 sec.)
4. Dry (5 sec.)
5. Dry (5 sec.)

Figure 2. Pin washing procedures. Default pin washing procedures (A) were modified (B) to insure complete removal of each oligonucleotide from the printing pin prior to loading the next oligonucleotide. While increasing the pin wash times increases the length of the print run, these procedures eliminate any carry-over of oligonucleotides from one feature to the next.

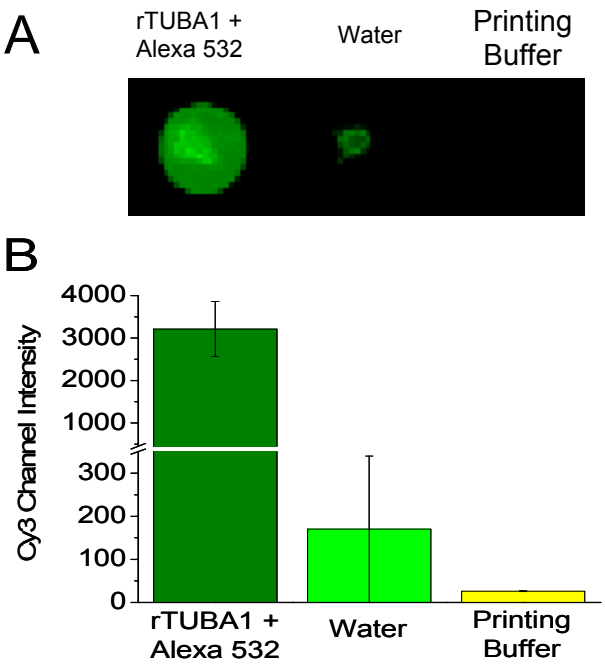

Figure 3. Carry-over of rTUBA1 during microarray printing. (A) Alexa 532-labeled oligonucleotide rTUBA1 (20 μM) was printed as the first tap on the slide (rTUBA1). After the pin was cleaned using the default wash procedure shown in Fig. 2A, the pin was dipped into water (no oligonucleotide) and then tapped onto the slide (Water). The pin was again cleaned and dipped into printing buffer before tapping on the slide (Printing Buffer). (B) The mean Cy3 channel signals from 4 arrays printed on 3 slides are shown. Using the wash procedure outlined in Fig. 2A, sufficient rTUBA1 remained on the pin to cause a carry-over Cy3 channel signal that was 5% of rTUBA signal.

Gene expression is often altered as a result of toxicant exposure and thus is a sensitive, measurable endpoint for toxicity that may serve as an early warning of compromised health. The challenges are to identify those genes that respond to toxicant exposure, to discover novel gene interactions, and to improve the knowledge of complex regulatory networks and cross-

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communication between different pathways during various chemical exposures. Microarray analyses provide a potential solution in that they measure the expression of thousands of genes simultaneously, providing data on the patterns of mRNA expression for most genes expressed in cells [6, 7, 10-12]. An important aspect of this technology is its use as a tool for the identification of molecular mechanisms of toxicity [13-15]. Such an approach enables researchers to identify single genes or whole genetic pathways that are involved in conferring resistance or sensitivity to toxic substances. Based on signature expression profiles of known toxicants, this technology holds promise to allow the characterization of unknown toxic compounds and an understanding of mechanisms of action of their toxicity [16-20].

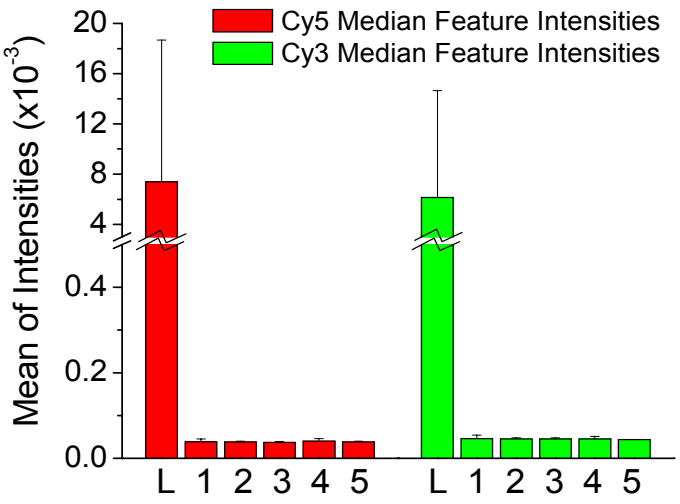

Figure 4. Oligonucleotide carry-over analysis. The means of the Cy3 and Cy5 channel feature intensities of the last printed oligonucleotide (L) of each of 16 subarrays is shown. In addition, the feature intensities of spots printed from the following 5 wells (which contained printing buffer alone) are shown (1-5). The modified pin washing procedure (Fig. 2B) eliminated any signal in the blank feature locations. This is data from a 4000 rat gene microarray hybridized with a rat tissue sample.

Typically, toxicologists have used rodent bioassays that require high doses, often take years to complete, and are expensive to identify potentially hazardous substances. This, coupled with traditional methods in molecular biology working on a "one gene at a time" basis, severely limits throughput for mechanism-based studies. By contrast, the analysis of the expression of thousands of genes in one experiment allows investigators to address important biological questions that have not been easily addressed with traditional expression-based technologies, such as Northern blots, *in situ* hybridizations, or RNase protection assays. Thus, DNA microarray technology, which can be used to analyze changes in genome-wide patterns of gene expression, is one new methodological advance that can dramatically accelerate the way toxicological problems are investigated [13].

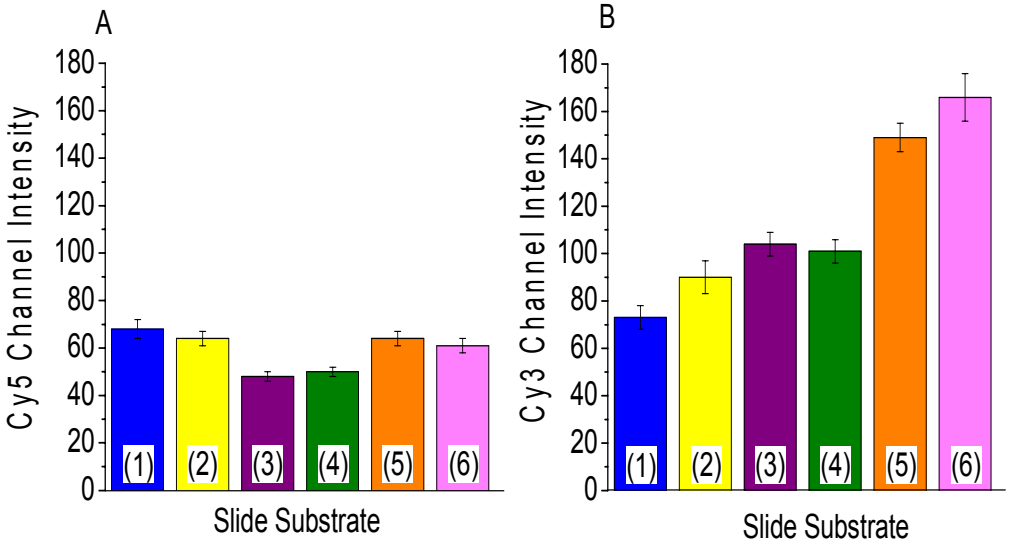

Figure 5. Background fluorescence from microarray substrates. Glass slides with the following substrate coatings were examined for background fluorescence in both the Cy5 (A) and Cy3 (B) channels. Microarray slides were: (1) in-house prepared poly-L-lysine coated slide; (2) poly-L-lysine coated slide from Erie Scientific; epoxy coated slide from MWG (3), GeneMachines (4), or Full Moon BioSystems (5); and aminosilane coated slide from Clontech (6). Slides were scanned using the Axon 4000B microarray scanner with laser power of 100% and photomultiplier gains set to 600. The data are means +/- SD of the median intensities of 20,160 spots (100 mm diameter) covering the printable area on each slide. Note different y-axis scales in (A) and (B).

The FDA anticipates the use of DNA microarray-based medical devices, as well as the submission of toxicogenomics data for support of investigational new drug applications (INDs), new drug applica-

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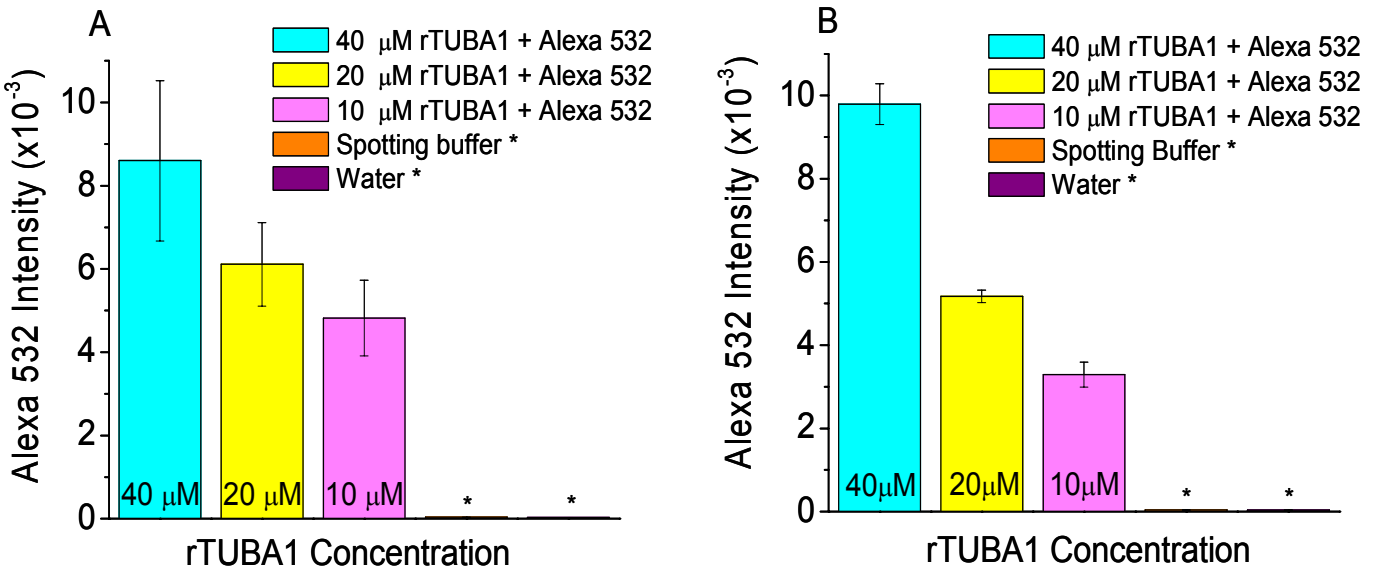

Figure 6. Feature intensity as a function of oligonucleotide concentration- poly-L-lysine coated slides. Alexa 532-labeled oligonucleotide rTUBA1 was printed onto poly-L-lysine coated slides from Erie Scientific at concentrations of 40, 20, and 10 μM. Following post-processing (A) or mock hybridization (B), the microarrays were scanned. When normalized to the 40 μM concentration, the ratios of Cy3 channel signal intensities were 1, 0.71, and 0.56 after post-processing and 1, 0.53, and 0.34 after mock hybridization. The spotting buffer and water (*) were at background levels.

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tions (NDAs), and biologics license applications (BLAs) [21]. This will create new challenges in the evaluation of such state-of-the-art technology. In response, the FDA has taken a proactive position by sponsoring workshops [22], publishing regulatory science perspectives [23], and creating draft guidance documents such as "Pharmacogenetic Tests and Genetic Tests for Heritable Markers; Draft Guidance for Industry and FDA Staff" (www.fda.gov/cdrh/oivd/guidance/1549.pdf). In November 2003, the FDA issued the "Draft Guidance for Industry: Pharmacogenomic Data Submissions" (http://www.fda.gov/cder/guidance/5900dft.pdf) to encourage the use of toxicogenomics data during drug development. This document outlines how and what data should be presented to FDA and how it will be used. In addition, the FDA is encouraging the voluntary submission of toxicogenomics data sets so the Agency can "be prepared to appropriately evaluate the anticipated future submissions" and so FDA scientists can develop an understanding of relevant scientific issues. The MicroArray Quality Control (MAQC) project, a large multi-institution collaboration involving FDA Centers, major providers of microarray platforms and RNA samples, EPA, NIST, academic laboratories, and other stakeholders, has also been recently implemented to provide quality control tools to the microarray community. The outcome of the MAQC project will be large publicly available reference datasets along with readily accessible reference RNA samples. These will be used to help avoid procedural failures and to develop guidelines for microarray data analysis. Complete information can be found at: (http://www.fda.gov/nctr/science/centers/toxicoinformatics/maqc/index.htm). Thus, the FDA is anticipating that pharmacogenomics data, including DNA microarray data, will become important information in the assessment of INDs, NDAs, and BLAs. Because of the complex nature of collecting such data and the potential high degree of variability introduced to the datasets by sub-optimal procedures and unrecognized sources of variability, observations and experiences with these issues are reported here. In addition, guidance is offered on how such problems can be overcome.

Fabrication of Microarrays

Oligonucleotide microarrays. Spotted oligonucleotide arrays exhibit a number of advantages over cDNA arrays [24, 25]. For example, oligonucleotides can be synthesized such that homologous sequences between genes can be excluded, thereby enhancing specificity. In addition, a given gene can be represented by a set of different oligonucleotides targeting different regions or exons, thereby allowing for the detection of splice variants, or the discrimination of closely re-

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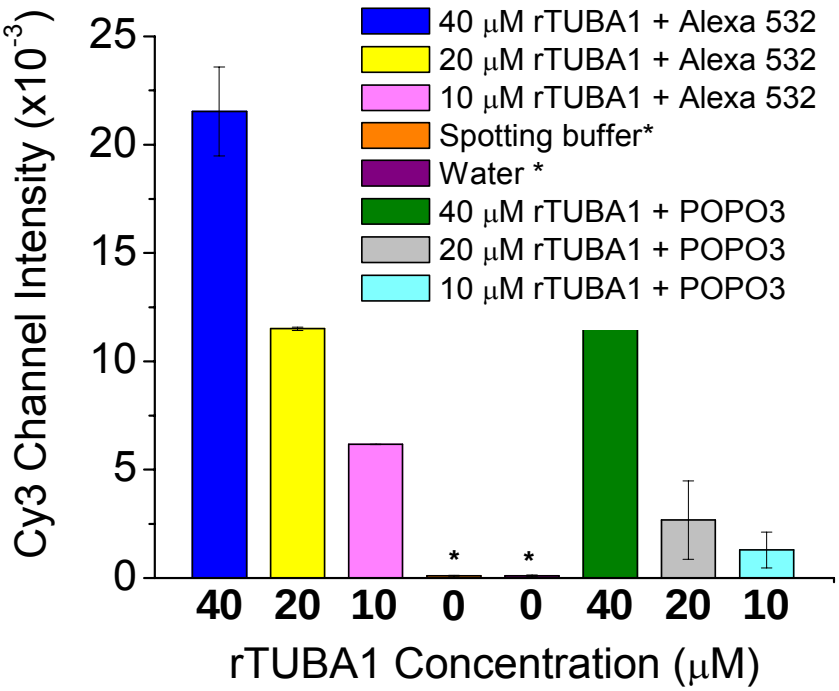

Figure 7. Feature intensity as a function of oligonucleotide concentration- aminosilane coated slides. Oligonucleotide rTUBA1, with and without covalently bound Alexa 532, was printed onto aminosilane coated slides from Clontech at concentrations of 40, 20, and 10 μM. Following post-processing, the microarrays were stained with the DNA dye POPO3 and scanned. When normalized to the 40 μM concentration, the ratios of Cy3 channel signal intensities are 1, 0.53, and 0.29 for the Alexa 532-labeled rTUBA1 and 1, 0.49, and 0.24 for the rTUBA1 stained with POPO3. The Cy3 signal from the spotting buffer and water (*) were at background

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lated genes, strains, or species. Also, cross-hybridization, which can severely mask true gene expression changes, is more of a problem with cDNA arrays than with oligonucleotide arrays. Oligonucleotide microarrays, therefore, offer potentially greater specificity and are an alternative to expensive cDNA library maintenance and amplification.

Microarray printing. The first step in conducting microarray experiments is to print (or spot) the oligonucleotide solutions onto a series of glass microscope slides that have been coated with a substrate capable of binding the oligonucleotides. Many variables (e.g., printing pins, printing buffers, pin washing procedures, oligonucleotide sources, slide substrates, temperature, and humidity) must be optimized to insure high quality arrays. Many of these variables have been examined in some detail for specific combinations of printing pins, printing buffers, temperature/humidity, etc. [26-29]. In the studies described here, all microarrays were printed using ArrayIt SMP3 printing pins (TeleChem International, Inc., Sunnyvale, Calif.) on the GeneMachines OmniGrid 100 printer (Genomic Solutions, Ann Arbor, Mich.). The ArrayIt SMP3 pins print oligonucleotides in a volume of 0.6 nl with diameters of approximately 100 μm. Rat, mouse, and human oligonucleotide libraries were purchased from BD Biosciences Clontech (San Jose, Calif.) and mouse oligonucleotides from MWG Biotech (High Point, N.C.). Either ArrayIt Micro Spotting Plus or MWG Spotting Buffer A printing buffers were used to print the oligonucleotides from 384-well polypropylene microplates. Satisfactory probe spots were obtained using GeneMachines OmniGrid 100 instrument settings of a dip time (the time the printing pins are immersed in the oligonucleotide solution) of 500 milliseconds and the minimum print time (the time the pins are in contact with the slide substrate). Following the dipping of the pins in the oligonucleotide solutions, the excess liquid is removed by "blotting" the pins using 12 consecutive taps at a spacing of 475 μm on a glass blot pad coated with poly-L-lysine. These instrument settings are adjustable and may need fine-tuning for various combinations of DNA, spotting buffer, slide surface chemistry, etc. After testing combinations of these parameters, the described settings became a standard for the present studies. Settings will also depend on the particular microarray printer used. The goal is to standardize as many aspects of the microarray process as possible in order to reduce variability.

Pin washing. Because each printing pin is used multiple times during a print run (410 times for printing 20,000 mouse oligonucleotides onto a glass microscope slide), every pin must be thoroughly cleaned after it prints each oligonucleotide onto the slides and before it dips into the next oligonucleotide solution. Any "carry-over" of oligonucleotide from one spot to the next would result in erroneous data. To assess potential oligonucleotide carry-over, a test oligonucleotide of 80 nucleotides (complementary to the rat tubulin gene; rTUBA1) was labeled with a fluorescent dye (Alexa 532) and was printed using the GeneMachines default wash procedure outlined in **Figure 2A**. These data (**Figure 3**) indicate a carry-over of rTUBA1 of approximately 5% and would present an error in gene expression data if this

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were a real experiment. To eliminate oligonucleotide carry-over, both the number of pin washes and the wash times were increased by over 4-fold (**Figure 2B**).

A method to determine if carry-over is a problem within a print run is available in the microarrays described here. When printing large collections of oligonucleotides from 384-well plates, the last plate is not completely filled with oligonucleotides; the remaining wells contain printing buffer only. Since the printer is not stopped until the contents of all the wells have been printed, there are blank features printed by each printing pin from the buffer-only wells. If carry-over exists, fluorescent signal would be detected in these blank features after hybridization. **Figure 4** shows an analysis of the median feature intensities, after hybridization, of the last oligonucleotide printed by each of the 16 printing pins plus the next five blank features. The data indicate that the modified wash procedure (**Figure 2B**) eliminates any oligonucleotide carry-over. Thus, probe carry-over contamination can be monitored for each printing pin on each microarray slide.

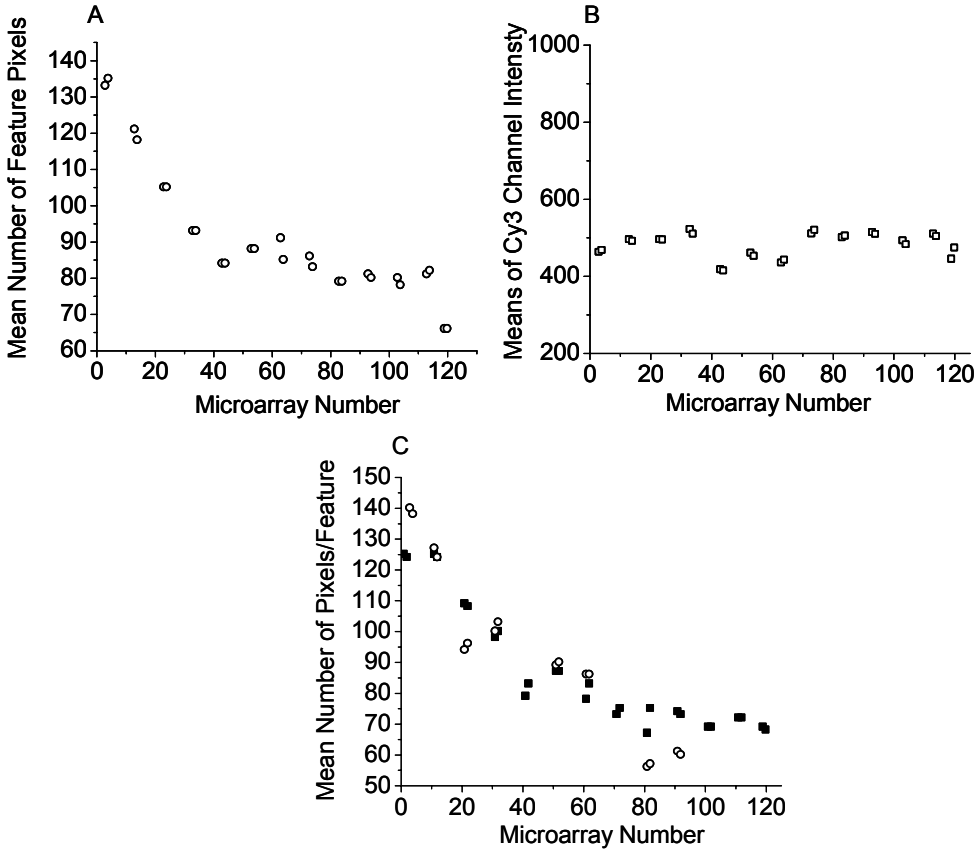

Figure 8. Feature size characteristics across a print run. Clontech rat oligonucleotides (20 µM) were printed on Erie poly-L-lysine coated slides in MWG Spotting Buffer A. Scans after post-processing were done using the Axon Instruments GenePix 4000B scanner with the laser power set at 100% and the PMT gains set at 600 for both the Cy3 and Cy5 channels. The "Find Irregular Features" option of GenePix Pro 5 software was used to produce the most accurate definition of feature boundaries. (A) The mean number of pixels per feature is presented as a function of the number of microarrays printed with one load of the printing pin. (B) The means of the median feature intensity are presented as a function of the number of microarrays printed. (C) Mean numbers of feature pixels after post processing (■) are shown along with the corresponding mean numbers of feature pixels after hybridization (○) as a function of number of microarrays printed.

Microarray slide substrates and background fluorescence. In construction of oligonucleotide microarrays, the probes must bind to a substrate (thin reactive coating) applied to a microscope slide. In addition to high levels of oligonucleotide binding, the substrate should be stable, have low inherent fluorescence, be free of localized optical anomalies, be cost-competitive, and offer ease of downstream processing of printed microarrays. An assessment of several microarray substrates produced in-house and from different commercial sources was conducted. These included poly-L-lysine produced in-house and purchased from Erie Scientific Co. (Portsmouth, N.H.); epoxy from GeneMachines, MWG, and Full Moon BioSystems, Inc. (Sunnyvale, Calif.); and aminosilane from Clontech. All of the slides were clear and free of surface defects; however, there were slight differences in inherent background fluorescence (**Figure 5**). While the signal from the Cy5 channel was quite low and uniform for all the slide types (**Figure 5A**), the signal from the Cy3 channel exhibited greater variability between slide types with the signal from the Full Moon BioSystems epoxy slides and the Clontech aminosilane slides significantly higher than those from the other slides. The background fluorescence from the Clontech aminosilane slides was approximately twice that of the poly-L-lysine coated slides (**Figure 5B**). Such background fluorescence will negatively impact the signal-to-noise ratio and result in reduced ability to detect significant gene expression changes. Similar findings have

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been reported by others [29].

Inactivating reactive sites on the poly-L-lysine slides after printing of the oligonucleotides (post-processing) can be accomplished using bovine serum albumin (BSA) as a blocking agent in an open laboratory environment. By contrast, the succinic anhydride post-processing procedure recommended for aminosilane coated slides requires 1-methyl-2-pyrrolidone, which must be used in a fume hood. Based on the scan data, ease of post-processing, and subjective evaluation of other criteria listed above, the poly-L-lysine slides exhibited low background fluorescence and were cost-effective for microarray experiments. Other combinations of slide substrates and blocking reagents may provide adequately low levels of background fluorescence, but it is important to find such combinations and standardize them in order to reduce variability.

Feature intensity as a function of oligonucleotide concentration. For microarray experiments to yield accurate and meaningful data, the oligonucleotide printed onto the substrate must be in excess of the amount of each individual labeled-cDNA applied during hybridization. Most microarrays are constructed using oligonucleotide solutions in the range of 20 µM to 50 µM. To assess the binding capacity of poly-L-lysine slides, various concentrations of a fluorescently labeled oligonucleotide (rTUBA1 as described above) were printed on the slides and the signal quantified. Micro Spotting Solution Plus (ArrayIt) spotting buffer and water were also printed on these arrays as negative controls. Microarrays were scanned after post-processing and after a mock hybridization, i.e., using hybridization buffer only with no labeled cDNA. **Figure 6** shows that relatively good proportionality exists between the amount of oligonucleotide printed and the feature intensity signal detected after post processing or after a mock hybridization. Little signal is lost during the hybridization.

In a separate experiment, rTUBA1, at concentrations of 40, 20, and 10 µm, with and without Alexa 532 covalently bound, was printed on aminosilane coated slides (**Figure 7**). Following post processing, these slides were stained with POPO-3 (Molecular Probes, Eugene, Ore.), a highly sensitive dimeric cyanine fluorescent nucleic acid stain, and scanned. These data also show a direct correlation between the amount of oligonucleotide printed onto the slide and the Cy3 intensity signal after post-processing. Thus, under these conditions, the binding of oligonucleotides to the substrate is proportional to the oligonucleotide concentration. Recently, it has been shown that there are significant DNA retention differences among slide types, and that the retention characteristics can decay with time [30]. Our method of evaluating the binding of oligonucleotides will be a useful tool for monitoring any changes in DNA binding and retention properties of slide substrates.

Quality control prior to hybridization. Because of the time, expense, and frequently limited amount of sample RNA available, quality control scans of all post-processed microarray slides are routinely conducted prior to using them for hybridizations. Analysis of the low level autofluorescence of the oligonucleotides (discussed in detail below) can be used as a tool to monitor feature characteristics,

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Sample [a]	RNA Integrity Number [b]						P [e]
	6/6/02 [c]	8/4/04	8/16/04	8/16/04	Average of 8/04 measurements	Difference [d]	
1A	7.5	7.5	7.7	7.5	7.6	-0.07	0.59
1B	7.6	7.0	7.1	6.7	6.9	0.67	0.01
1D	8.1	7.7	8.0	7.8	7.8	0.27	0.17
2B	7.9	7.8	8.0	7.7	7.8	0.07	0.41
2D	8.4	7.9	8.5	7.7	8.0	0.37	0.10
3A	8.5	7.9	8.2	7.7	7.9	0.57	0.03
3B	8.2	8.1	8.5	8.1	8.2	-0.03	0.55
3D	7.1	7.6	-	7.4	7.5	-0.40	0.91
4B	7.8	7.2	-	7.3	7.3	0.55	0.04
4C	7.7	7.2	7.6	7.2	7.3	0.37	0.10
4D	7.8	7.1	7.7	7.1	7.3	0.50	0.04

Table 1. Stability of RNA stored in water at -80º C

[a] RNA was isolated using the TriReagent method from individual rat livers, and the RNA was dissolved in sterile water and stored in small aliquots at -80º C.
[b] RNA Integrity Number (RIN) was assigned by the Agilent Bioanalyzer software and is a measure of RNA quality as described in the text.
[c] Date of evaluation of RNA.
[d] Difference between the average of the 8/04 RIN values and the original 6/6/02 RIN values.
[e] P-values tested a one-sided hypothesis that the original RNA has a higher RIN score than the RNA stored in water for more than 2 years.

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as well as the general quality of the microarrays prior to hybridization. Slides that have dust particles, within or closely adjacent to oligonucleotide features, or that show evidence of possible substrate separation are discarded. This insures that RNA samples and hybridization reagents are not wasted due to a defective or substandard microarray. **Figure 8A** shows the mean numbers of pixels per microarray feature during a print run of 120 microarrays. As can be seen, there is an approximately 40% decrease in the number of pixels per feature area during a print run. Despite this reduction in the number of pixels per feature, the median feature pixel intensities remain constant (**Figure 8B**), indicating that while the absolute amount of oligonucleotide deposited on the slide decreases, the density of the oligonucleotide on the slide remains constant. **Figure 8C** shows a close correspondence between the number of pixels per feature before and after hybridization. These analyses indicate that, while feature size decreases during a print run, it does not affect the outcome of the data analysis based on median feature intensity.

Samples	Reaction components			Dye incorporated into cDNA (pmol) Cy3/Cy5	Nucleotide/ dye ratio in cDNA **Cy3/Cy5**
	Amount of total RNA (µg)	aa-dUTP/ dTTP	Cy dyes (pmoles)		
A / B[a]	10 µg	2:3	6,250	226 / 306	80 / 63
A / B	10 µg	2:3	20,000	300 / 302	54 / 57
A / B	10 µg	7:3	20,000	544 / 342	29 / 50
A / B	10 µg	7:3	40,000	343 / 321	44 / 49
A / B	20 µg	7:3	40,000	380 / 266	53 / 77

Table 2. Dye incorporation into cDNA

[a] Sample A (rat liver RNA-1) labeled with Cy3 and sample B (rat liver RNA-2) labeled with Cy5.

RNA Preparation

High quality RNA samples are key to obtaining reliable microarray data. An otherwise successful microarray experiment will be completely invalidated by beginning with samples of degraded RNA. It has been demonstrated that up to three-quarters of differential gene expression can be due solely to differences in RNA integrity between samples [31]. Another study confirms the decrease in data quality obtained from degraded sample RNA, although it is suggested that "moderate" degradation may yield usable results [32]. Several widely used RNA isolation methods were investigated for the purification of intact RNA from the livers of mice, including TriReagent [33] (Molecular Research Center, Inc., Cincinnati, Ohio), FastRNA Pro Green Kit for animal tissue (Qbiogene, Inc., Carlsbad, Calif.), and Qiagen RNeasy Kit (Qiagen Inc., Valencia, Calif.). Pieces of fresh liver tissue rapidly removed from humanely euthanized mice were used in all procedures, and RNA was extracted from three tissue samples by each method. The tissue samples processed with TriReagent and the RNeasy Kits were disrupted using the manufacturers recommended conditions with a motor-driven Teflon pestle in a tight-fitting centrifuge tube. The tissue samples processed with the FastRNA Kit were disrupted with the FastPrep cell disruptor (Qbiogene, Inc., Carlsbad, Calif.), which uses a high-speed reciprocating device to propel small beads into the tissue. After RNA isolation, the RNA quality was assessed by capillary electrophoresis using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, Calif.). As can be seen in **Figure 9**, the nine samples were all of similar high quality with little degradation. The Bioanalyzer uses software to assess the quality of the RNA based on the electrophoretic tracings and calculates an RNA Integrity Number (RIN) that ranges from 10 for "perfect" intact RNA to 1 for completely degraded RNA. The average RIN was 9.0, with all except one sample being > 8.4. The RNA degradation software (Degradometer) [31] was also evaluated, and a good correlation was found between the RIN values and the Degradometer values (R=0.8). Thus, each method can produce high quality RNA. The Qiagen kit provided advantages of ease of use and consistency.

RNA samples dissolved in sterile, RNase-free water are often conveniently stored at -80º C until use. Extended storage of RNA under these conditions may result in degradation of the RNA. To investigate this, RNA samples were evaluated using the Agilent Bioanalyzer before and after more than two years under these conditions. **Table 1** shows that the integrity of 4 of 11 samples was significantly reduced and, that overall there was a significant decline in the average RIN

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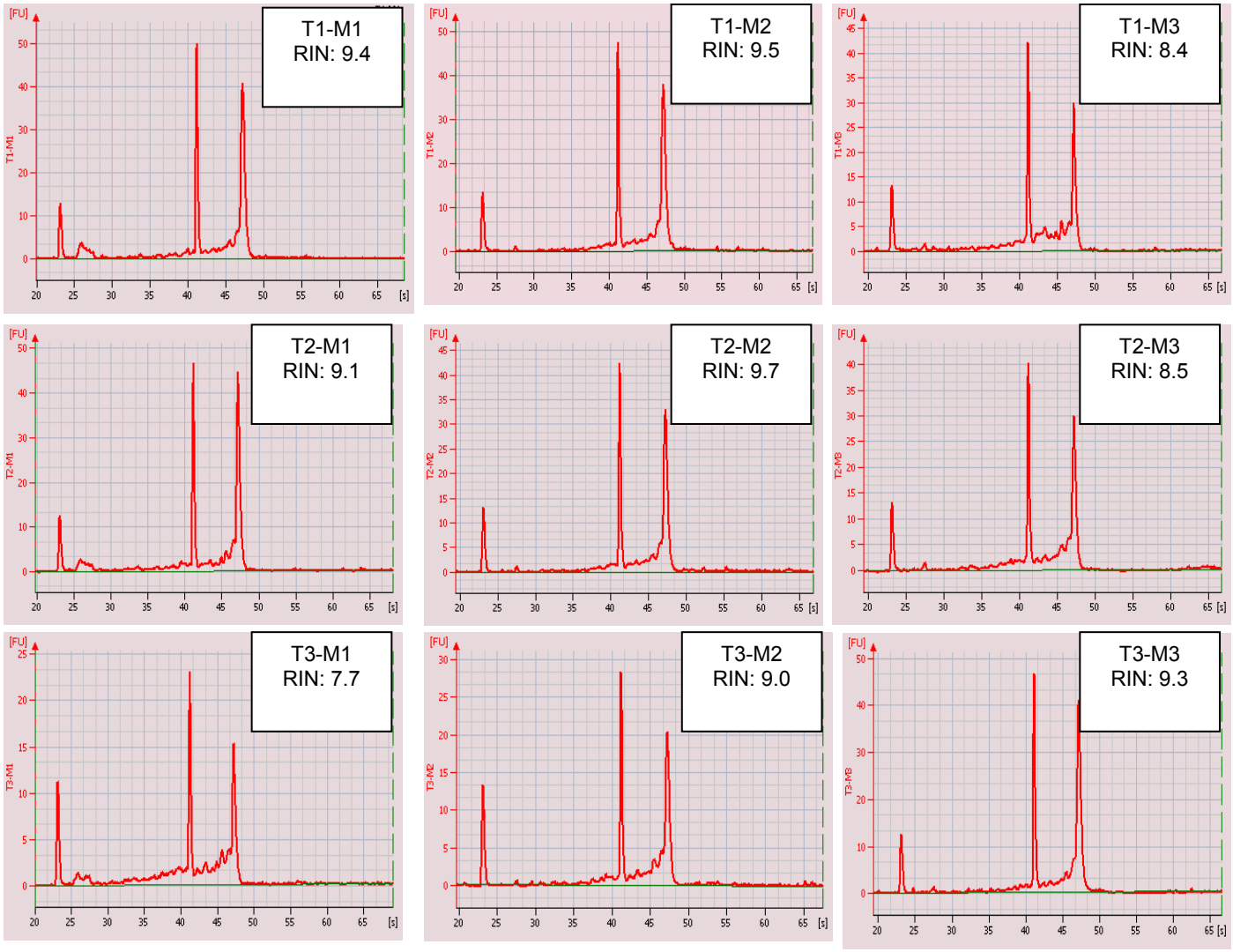

Figure 9. Comparison of RNA isolation procedures. RNA was purified from 3 mouse livers (T1-T3) by three different methods (M1: FastPrep; M2: Qiagen RNAeasy kit; M3: TriReagent) as described in the text. The integrity of the RNA was evaluated using the Agilent Bioanalyzer. Electrophoretic tracings are shown along with the RNA integrity number (RIN).

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value of 0.26 ($p = 0.01$). While significant, this relatively modest reduction in RNA quality, over a period of two years, suggests that storage of RNA under these conditions for relatively short periods of time may not negatively impact microarray results.

Preparation of Labeled Targets

Aminoallyl concentration and dye incorporation. The indirect labeling of sample RNA involves the production of cDNA containing amino groups through the inclusion of aminoallyl-dUTP (aa-dUTP) in the reverse transcription reaction. In a subsequent reaction, the amine-reactive fluorescent molecules, cyanine-3 (Cy3) or cyanine-5 (Cy5) [34], are attached to the incorporated amino groups in the cDNA creating the labeled target molecules. Recent studies have described various characteristics of this labeling scheme and have suggested optimal labeling densities and reaction conditions [35-37]. Even though these guidelines exist, it is important to optimize the labeling reactions in an individual laboratory under site-specific conditions. Starting with total RNA from the samples, the effects of the ratio of aa-dUTP to dTTP, the amount of RNA in the reverse transcription reaction, and the amount of reactive fluorescent dyes were examined. **Table 2** shows that highly fluorescent targets can be created from 10 µg of total RNA, with the highest specific activities (low nucleotide to dye ratios) obtained by using more aa-dUTP and large amounts of reactive dyes. When hybridized to microarrays, it was found that a ratio of aa-dUTP to dTTP of 2:3, and 6,250 pmoles of reactive dyes re-

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sulted in acceptable signal-to-noise ratios and was cost effective.

Quality of purification columns. The synthesized aminoallyl-cDNA was purified with the widely used QIAquick PCR purification columns (Qiagen, Valencia, Calif.) before labeling with the reactive dyes. These columns were then used to remove uncoupled fluorescent dye from the fluorescently labeled cDNA. Therefore, the quality of the columns is a critical factor as faulty columns might result in insufficient recovery or purity of the sample loaded on the column. An experiment was carried out where cDNA synthesized from six RNA samples was pooled together, split into six samples, and passed through three columns each from two different QIAquick PCR purification kits with different lot numbers. Use of one lot of columns resulted in losses of greater than 30% of the cDNA (data not shown).

In another experiment, samples with known amounts of cDNA were passed through columns to check the efficiency of recovery from the columns. **Table 3** shows that one lot of columns resulted in poor and highly variable recovery of the cDNA (samples 1-5; recovery ranged from 15% to 73%), while the use of columns from a different lot resulted in good recovery of the cDNA (samples 6-9; 84% to 90%). Such poor recovery of cDNA would ultimately affect the quality of the microarray. Thus, these widely used purification columns can be the source of significant loss of cDNA that will result in reduced signal-to-noise ratios. These data also indicate the importance of monitoring cDNA yield at the end of the synthesis and labeling procedures.

These results clearly indicated that the quality of the purification columns is very important for consistent and high-level recovery of cDNA, as well as fluorescently labeled cDNA. Therefore, before initiating any microarray experiment, it may be necessary to perform a pilot experiment with cDNA samples to determine the quality of the purification columns. Indeed, this illustrates the need to institute quality control criteria on all aspects of the microarray procedure.

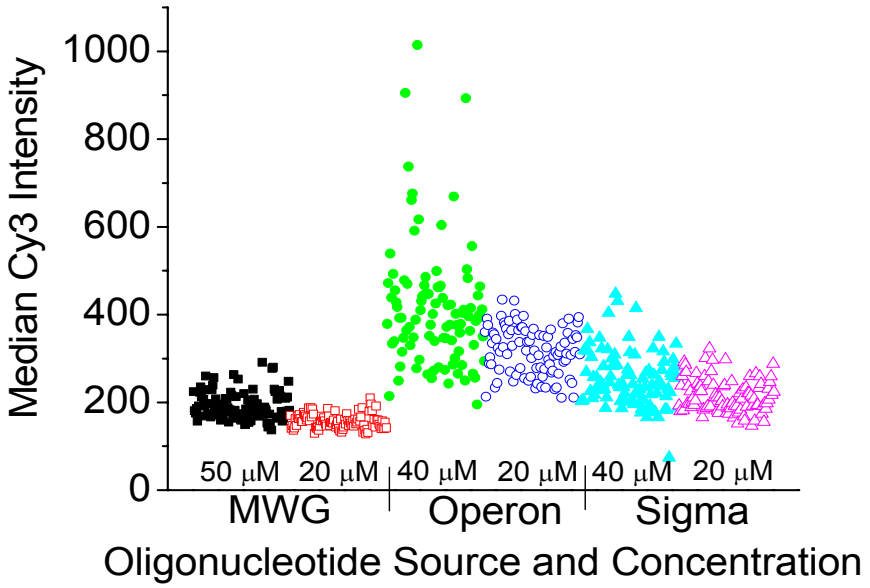

Figure 10. Autofluorescence of features. Approximately 96 oligonucleotides from each of three manufacturers were printed at two concentrations on the same poly-L-lysine coated slides. The Cy3 channel median intensity (an indication of autofluorescence) of each feature is shown. The features are approximately 100 μm in diameter. Slides were scanned using the Axon 4000B microarray scanner with laser power of 100% and PMT gains set to 600.

Sample [a]	cDNA (pmol)		Recovery (%)
	Expected	Observed	
1	18020	9557	53
2	17274	12697	74
3	20072	8045	40
4	17267	2538	15
5	16960	8695	51
6	13491	11363	84
7	20024	17247	86
8	14613	12978	89
9	20230	18266	90

Table 3. Effect of columns on the recovery of cDNA

[a] cDNA samples 1-5 were passed through a single lot of QIAquick PCR Purification columns while samples 6-9 were passed through a different lot of columns.

Effect of dimethylsulfoxide on dye coupling. As described above, aminoallyl-cDNA was labeled with either Cy3 or Cy5 fluorescent molecules. Cyanine dyes provided in lyophilized form were suspended in dimethylsulfoxide (DMSO) prior to labeling reactions. Dimethylsulfoxide is hydroscopic in nature and, as cyanine dyes are rapidly hydrolyzed in water, it is important to use DMSO that has not been exposed to humid air. The effect of DMSO on dye coupling was examined by using DMSO from newly opened vials and DMSO that had been opened and used in the laboratory environments, and then stored in a desiccator for several months. **Table 4** shows a profound reduction in dye incorporation, as evidenced by a poor nucleotide/dye ratio, as a result of using "old" DMSO compared to freshly opened "new" DMSO. From these results, it is

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evident that the quality of DMSO is a major factor in generating highly fluorescent targets.

Amount of dyes for optimal signal intensity. Several properties of the fluorescently labeled cDNA can be determined from spectrophotometric analysis before these targets are used on a microarray slide. Absorbance at 260 nm can be used to calculate the amount of cDNA, and the absorbance of the Cy3 and Cy5 at their absorption maxima can be used to calculate the amount of the dyes incorporated into the cDNA [38]. Thus, the overall amount of label in each sample, as well as the nucleotides of cDNA to dye ratio (a measurement similar to specific activity in radioactive probes) can be calculated. The amount of dye incorporated into samples used within an experiment should be consistent in order to minimize variability. It would be expected that the higher the dye concentration in the target samples, the brighter the signal on the microarray. An experiment was carried out in which one set of microarrays was hybridized with samples containing 50 pmoles each of Cy3 and Cy5 dye, and another set of microarrays was hybridized with samples with dye incorporation of more than 200 pmoles each of Cy3 and Cy5 dyes. These microarrays were scanned on a Genepix 4000B scanner with balanced PMTs. The average signal/noise ratio for microarrays hybridized with more than 200 pmoles of fluorescent dyes was 24.2 for Cy3 and 12.6 for Cy5. This was more than 2.5 times higher for both Cy3 and Cy5 channels than the signal/noise ratio for arrays hybridized with 50 pmoles of dyes. This allows the detection of lower expressing genes and permits the more accurate analysis of the microarray data.

Sample [a]	Old DMSO				New DMSO			
	Dye incorporation (pmol)		Nucleotide/dye		Dye incorporation (pmol)		Nucleotide/dye	
	Cy3	Cy5	Cy3	Cy5	Cy3	Cy5	Cy3	Cy5
1	102	-	121	-	202	-	72	-
2	114	-	114	-	200	-	82	-
3	80	-	230	-	288	-	64	-
4	49	-	327	-	276	-	65	-
5	86	-	224	-	249	-	69	-
6	-	149	-	96	-	243	-	57
7	-	171	-	78	-	217	-	61
8	-	64	-	235	-	241	-	71
9	-	97	-	205	-	195	-	77
10	-	73	-	237	-	192	-	67

Table 4. Effect of DMSO on dye incorporation

[a] Samples were RNA from mouse liver and from a mouse lymphoma cell line. Two 10-µg aliquots of each RNA were converted to cDNA, and one was labeled with Cy dyes dissolved in "old" DMSO and the other labeled with Cy dyes dissolved in "new" DMSO.

Autofluorescence

Autofluorescence of features as a factor in microarray quality. Autofluorescence, defined as the presence of feature-originating signal in the absence of fluorescing dye from cDNA targets, is commonly observed in microarrays and is commonly found only in the Cy3 channel [39, 40]. It is often of unknown origin and has a slightly different fluorescence spectrum than Cy3 [39]. This problem can cause a decrease in sensitivity of fluorescence detection in that channel and can lead to erroneous intensity values and ratios in low intensity features. This can be problematic in flip-dye experiments because there will always be substantial amounts of fluorescence in the Cy3 channel, in addition to the gene-specific contribution from the labeled cDNA. Studies have shown that pre-hybridization scans cannot be used to predict the post-hybridization autofluorescence of the features because of the dramatic variation seen across individual arrays [39]. Therefore, this potential problem should be investigated, avoided, and/or corrected when possible. If the problem goes unrecognized, it will mask true gene expression changes.

Substantial autofluorescence was noted on slides printed with rat, mouse, and human oligonucleotide collections from Clontech when the genes were printed in either Micro Spotting Solution Plus buffer (ArrayIt) or Printing Buffer A (MWG). Intensities of the spots varied over an order of magnitude, from approximately 100 to >2000 relative fluorescent units (rfu) under standard scanning conditions. In contrast, blank features (buffer only) assessed in the Cy3 channel and all features analyzed in the

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Cy5 channel exhibited relatively constant intensities with an average feature intensity of 98 rfu with a background slide fluorescence of 91 rfu. After hybridizing these slides under standard conditions in the absence of labeled cDNA (mock hybridization), the autofluorescence signal in the Cy3 channel was reduced by about half. The Cy5 feature intensity remained slightly above background with a mean intensity of 72. This level of autofluorescence on the spots prevents the accurate measurement of low expressing genes.

To determine the source of this problem, the printing buffer, the water used to dilute the printing buffer, the Seal & Sample Aluminum foil lids used to seal oligo plates for storage, and oligo source were considered as possible causes of the contaminating feature fluorescence. The data in Figure 6 indicate that neither printing buffer nor water contributed significantly to the autofluorescence.

Another potential source of autofluorescence was the possible adsorption of organic molecules, or some other substance, into the oligo solution from the adhesive Biomek Seal & Sample Aluminum foil lids (Beckman, Fullerton, Calif.) used to seal the 384-well plates used during freezer storage. To test this possibility, DNA solutions were spotted onto the foil for varying lengths of time and then spotted onto slides. Additionally, slides with spotted arrays were sealed in chambers containing the adhesive aluminum foil for varying lengths of time. There was no increase in fluorescence, indicating that the adhesive foils were not the source of feature-localized autofluorescence.

Oligonucleotides from three different suppliers were examined for feature-localized autofluorescence. Sample plates were obtained from: MWG Biotech AG (Ebersberg, Germany), Operon Technologies (Valencia, Calif.), and Sigma Genosys (Woodland, Texas). Each plate of oligos was reconstituted with printing buffer and printed at two concentrations sequentially on the same slides for evaluation. The MWG oligos were printed at 50 µM and at 20 µM. The Operon and Sigma oligos were each printed at 40 µM and at 20 µM. After printing, the slides were scanned on the Axon 4000B scanner at 600 PMT setting with 100% laser power (typical setting for microarray scanning). The Cy3 intensity for each feature was plotted against feature position on the printed slide (**Figure 10**). These experiments indicated that MWG oligos had the least autofluorescence with a mean Cy3 feature intensity of 192 rfu at 50 µM and 156 rfu at 20 µM. The mean Cy3 feature intensity for the Operon oligos was 414 rfu at 40 µM and 316 rfu at 20 µM. The mean Cy3 feature intensity for the Sigma oligos was 256 rfu at 40 µM and 215 rfu at 20 µM. In conclusion, one evaluation measure that can be used in combination with other performance criteria when considering the purchase of an oligo library may be the amount of feature-localized contaminating autofluorescence.

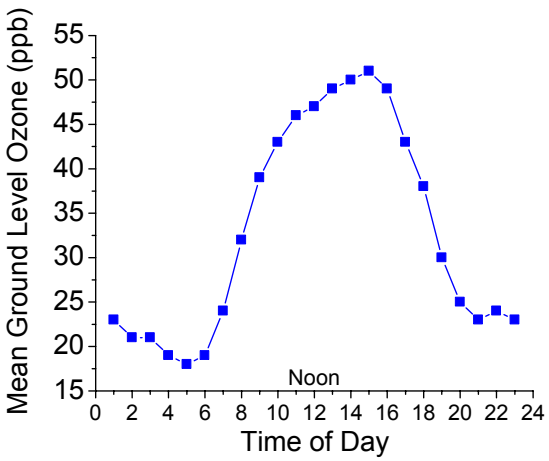

Figure 11. Diurnal ozone fluctuations. The means of hourly atmospheric ozone levels measured during a 22-day period for July 2004 in central Arkansas by the ADEQ are shown. Peak ozone concentrations averaged between 46-51 ppb between 11:00 a.m. and 4:00 p.m. Ozone levels during this period commonly reach into the 60-70 ppb range and can reach as high as 89 ppb.

The actual cause of feature localized autofluorescence is unknown, is often unavoidable, and can be variable. However, it can be reduced significantly by chemical means. Sodium borohydride, a reducing agent, has been used to reduce autofluorescence from undetermined sources on microarrays [40]. Two protocols [40, 41] using sodium borohydride to reduce the autofluorescence on in-house microarrays were evaluated. Slides were scanned before and after each treatment. Results from this study showed that both methods were effective in reducing the amount of autofluorescence, with the Raghavachari method producing a mean percent decrease of 55.6 ± 5.5 and the Massimi method producing a mean percent decrease of 44.2 ± 7.3. By reducing the autofluorescence of the features, these methods may help to increase the sensitivity of fluorescence detection in the Cy3 channel. While sodium borohydride use, in conjunction with oligonucleotide collections exhibiting high autofluorescence, will improve detection of low expressing genes, it is probably not necessary for oligonucleotide collections exhibiting low levels of autofluorescence.

Hybridization

Automated hybridization instruments. Conventional microarray hybridization is accomplished by sandwiching the sample hybridization solution between a coverslip and a DNA microarray, forming a capillary gap. The hybridization process relies on diffusion as the only means of mixing of labeled cDNA with surface-bound probes. The calculated movement of a tar-

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System [a]	Condition	Cy5 channel median CV (%)	Cy3 channel median CV (%)	Cy5 channel mean S/N	Cy3 channel mean S/N
A	Instrument	19.8	24.6	10.7	17.8
	Manual	17.4	12.2	3.2	8.3
B	Instrument	15.9	12.7	14	19
	Manual	26	24.5	10	24
C Experiment 1	Instrument	16	13	3	4
	Manual	13.5	8.5	2	4
C Experiment 2	Instrument	21.5	18.5	9	8
	Manual	14	8	3	2
D Experiment 1	Instrument	14.5	11.8	16	24
	Manual	14	10.5	8	13
D Experiment 2	Instrument	19.5	16	12	20
	Manual	28	20	4	7

Table 5. Median CV and signal-to-noise (S/N) ratios for automated and manual hybridization

[a] Automated hybridization instrument is described in text.

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get molecule due to diffusion over an 18-hour hybridization is less than 1 mm [42]. Therefore, each arrayed spot is sampling only from its immediate environment in the absence of mixing, possibly leading to depletion of targets resulting in inaccurate signal detection. To achieve the maximum specificity and sensitivity potential of a microarray and to decrease intra-slide variability and nonuniformity, it may be necessary to incorporate mixing during hybridization.

There are a number of commercially available and prototype hybridization instruments that incorporate a variety of technologies to accomplish sufficient mixing to overcome the diffusion limitation. Four commercially available hybridization systems were evaluated and compared to the currently used manual microarray hybridization. The first two instruments (A and B) that were evaluated were completely automated systems that completed both the hybridization and post-hybridization wash steps; the second two instruments (C and D) provided only for the completion of the hybridization step in the instrument and required completely manual post-hybridization washes. Demonstration instruments for three of the four instruments (B, C, and D) were set up by their respective vendors for in-house testing. Instruction on how to use each was also given by vendor representatives. Instrument A (discussed below) is large and costly and was not available for in-house testing, so the instrument testing was completed in the vendor's laboratory.

System A uses a patented fluid-like coverslip that creates and maintains a humid reaction chamber to prevent evaporation. To facilitate mixing, a stream of air is aimed at the liquid coverslip, and this creates a vortex in the aqueous hybridization solution underneath, promoting uniform mixing. To evaluate this system a stock of labeled cDNA was sent with in-house prepared microarrays to the company for hybridization using the instrument. Hybridization and wash condition information was also sent to the company for use with the system. A portion of the stock labeled cDNA was also kept in-house for completion of manual hybridizations for comparison. System B uses a temperature-controlled chamber for hybridization and washing. Hybridization solutions are pumped back and forth through the hybridization chamber to facilitate mixing. There were difficulties with chamber leakage during hybridization and/or slide washing, which created a problem with background and lack of hybridization uniformity on some slides. System C uses acoustic waves to agitate the sample solution during hybridization [43]. This technology uses a customized agitation chip attached to a glass carrier in lieu of a coverslip. System D uses two air-driven bladders contained in the custom made coverslip to provide hybridization mixing [44].

Systems B, C, and D were compared to in-house manual hybridizations. To evaluate each of the systems, a stock of labeled cDNA was made for each experiment and used to compare the automated and manual hybridizations. A 5,082 oligonucleotide array, printed in duplicate on each slide, was used for each comparison experiment. After hybridization and washing, slides were scanned, and the median intensity coefficient of variation (CV) for both the Cy3 and Cy5 channels was compared between the duplicate arrays for each slide. The mean signal-to-noise (S/N) ratio was also compared.

As shown in **Table 5**, none of the instruments offered much improvement in CVs over the manual

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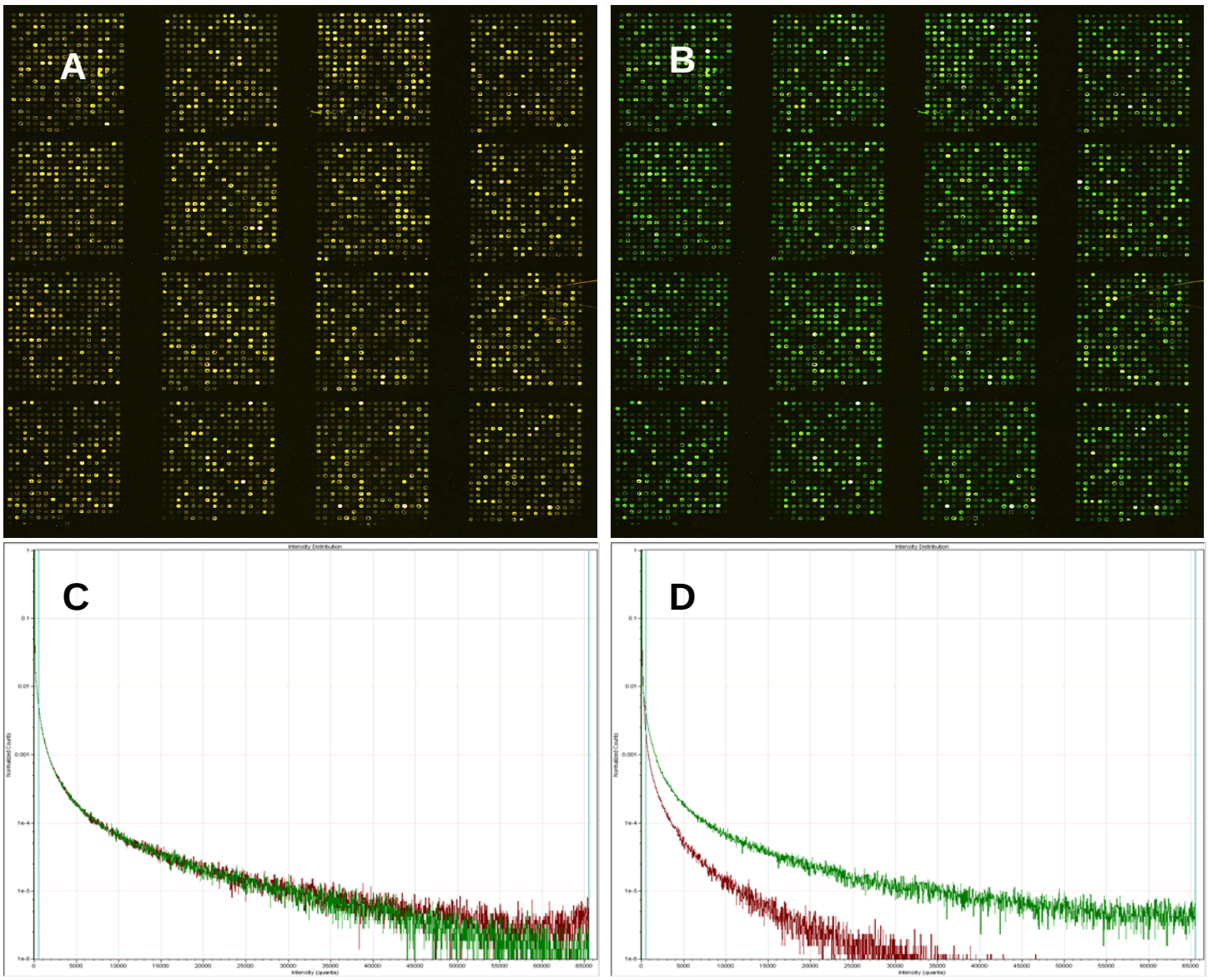

Figure 12. Ozone-exposed microarrays. Microarray images before (A) and after (B) a 5-hour exposure to ambient ozone are shown. Reduction in Cy5 causes the microarray image to have a predominantly green cast. Corresponding histograms of signal intensities (C and D) illustrate that the distribution of intensities in the Cy5 and Cy3 channels are very different after ozone exposure. Both scans were performed using the same scanner settings.

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hybridization method, although some of the instruments reduced the intra-slide variability. There was often, however, a large improvement in the signal-to-noise ratio (presumably due to the mixing that each one provided), which would allow evaluation of low expressing genes. Because of time and other limitations, the performance of each instrument could not be extensively fine-tuned, and this may have been the reason for leakage from some instrument chambers. It is, therefore, possible that each system may have the potential for improved performance. In theory, automating the hybridization and wash procedures would be expected to reduce overall variability.

Ozone as a factor in microarray quality. Ozone (O_3) oxidation of cyanine dyes, such as the Cy5 dye molecule, has been identified as a cause of reduced Cy5 signal intensities in microarrays [45]. Specific degradation of the Cy5 signal would, in turn, result in inaccurate gene expression ratios (e.g., Cy5/Cy3) and erroneous interpretation of microarray data. Ground level ozone is present at all times of the year but is generally higher during the summer months. Ozone is also higher in urban and/or industrialized

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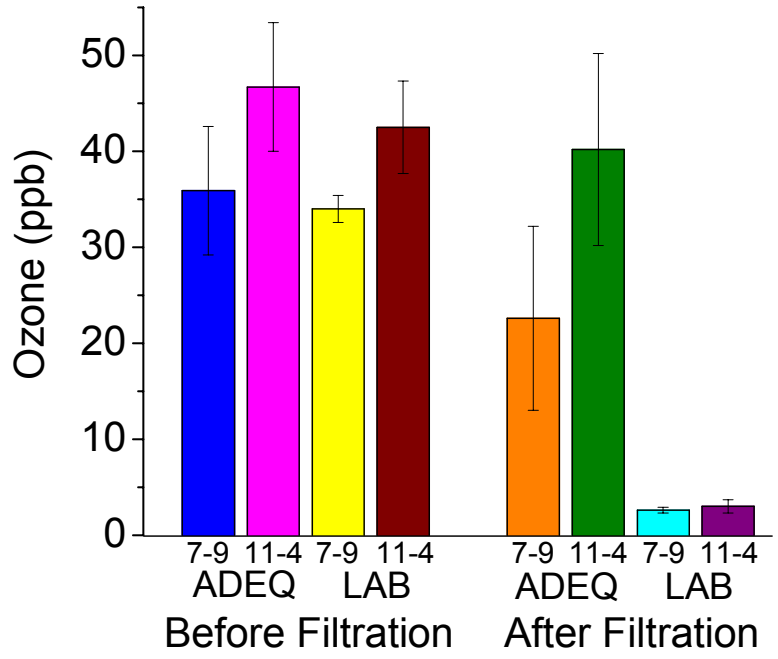

Figure 13. Laboratory ozone levels. Ozone was measured in the microarray laboratory for 10-day periods before and after HVAC modifications were completed. Ozone was reduced from 34-42 ppb before filtration to 2-4 ppb after filtration. Ozone levels measured by the ADEQ in central Arkansas during the period corresponding to the after-HVAC modification time averaged between 22 ppb to 47 ppb.

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areas as a principal component of smog. The presence of hydrocarbons and nitrogen oxide pollutants from automobile exhaust and factory emissions, when combined with exposure to sunlight in the presence of little air movement, leads to the generation of ozone in the lower atmosphere. Ozone levels as low as 5-10 ppb are capable of oxidizing Cy5 [45]. Ozone levels measured in central Arkansas between July 1 and July 22, 2004, averaged 33 ppb but reached a high of 89 ppb during this interval (Arkansas Department of Environmental Quality (ADEQ); (http://www.adeq.state.ar.us). In addition to seasonal changes, a diurnal variation of ozone levels occurs because more ozone is produced as the atmosphere heats during daylight hours (**Figure 11**). These data indicated that a peak of ozone levels occurs between approximately 11:00 a.m. and 4:00 p.m.

The problem of microarray deterioration became apparent when the red fluorescence signal seemed to disappear during the scanning process. To follow this up, histograms of pixel intensity frequencies from oligonucleotide microarray scans, done at two time intervals, were examined and found to be dramatically different (**Figure 12**). The Cy5/Cy3 intensity ratio of this microarray was initially balanced at 1.02. When the microarray was rescanned six hours later, under the same instrument settings, the Cy5/Cy3 intensity ratio was reduced to 0.33. This change in Cy5/Cy3 intensity ratio was primarily due to the reduction in Cy5 intensity as can be seen by comparison of **Figures 12A** and **12B**. However, we have also noted a small increase in Cy3 intensity during these experiments.

An attempt to minimize degradation of the Cy5 dye due to the "ozone effect" prompted three changes in the scanning protocol. Firstly, microarray hybridization experiments are scheduled so that the washing and scanning occurred early in the morning hours (7:00-9:00 a.m.) when ozone levels were the lowest. Secondly, the microarray slides are scanned **immediately** after the final wash. This was done by centrifuging the slide dry for 10 seconds and placing it directly into the scanner. Thirdly, the installation of carbon filters in the heating, ventilation, and air conditioning (HVAC) supply ducts in the microarray laboratory dramatically reduced laboratory ozone. The process for adding carbon filtration to our existing HVAC system involved several steps. Jefferson Laboratories engineers consulted experts at Agilent Technologies, who helped select an appropriate carbon loaded nonwoven filter; a HEGA filter series 2653, which is a 24" x 24" x 12" carbon filter and which has a low pressure drop of 0.3 inches (water column) at the maximum air flow rate of 1,100 cubic feet per minute. Because of the low pressure drops, these carbon filters can be successfully installed in many low pressure systems provided that enough space exists in the filter housing. The laboratory was pressurized relative to the corridor to prohibit nonfiltered air from entering the microarray laboratory. To reduce particulates in the laboratory and prolong the life of the carbon filter from three months to one year, 95% efficient filters were installed in the central air handler to replace the existing 35% efficient filters.

It is perhaps important to note here that another possible solution to the ozone problem was assessed, i.e., purchasing a separate enclosure to be constructed within

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the microarray laboratory to exclude ozone. These units contain flexible side panels supported by an aluminum frame with an ozone-filtered air supply at the top of the unit. However, the cost of the HVAC modifications to remove ozone from the laboratory supply air by carbon filtration was about one-half of that incurred for the purchase of the ozone-filtered enclosure within the lab. In addition to the cost savings, the entire laboratory is ozone free as opposed to the small space enclosed by the ozone-free enclosure system.

Reduction of ozone in the laboratory by carbon-filtering the supply air was dramatic (**Figure 13**). Ozone levels were measured using a Model 450 Ozone Monitor (Advanced Pollution Instrumentation, Inc., San Diego, Calif.) and recorded using software constructed on site. Ozone measurements during a 10-day period before the modifications were begun averaged between 34 and 42 ppb during the morning (7 a.m. to 9 a.m.) and during the period of peak ozone levels (11 a.m. to 4 p.m.), respectively. These readings are in line with the ozone readings taken in central Arkansas by the ADEQ (42 ppb). In contrast, laboratory air after ozone filtration ranged between 2.6 and 3.0 ppb compared with an average of 35 ppb at peak time outside.

Using these microarray scanning procedures and engineering modifications, ozone degradation of the Cy5 dye was minimized (**Figure 14**). In this experiment four microarrays were initially scanned in the ozone-filtered laboratory. After scanning, two of the microarrays were moved to the adjacent lab without ozone-filtered supply air. All four slides were then rescanned at 1-hour and at 6 hours. The data indicate a 3% reduction of Cy5 intensity after one hour in the ozone-reduced laboratory but a 55% reduction in the nonozone-filtered laboratory. After

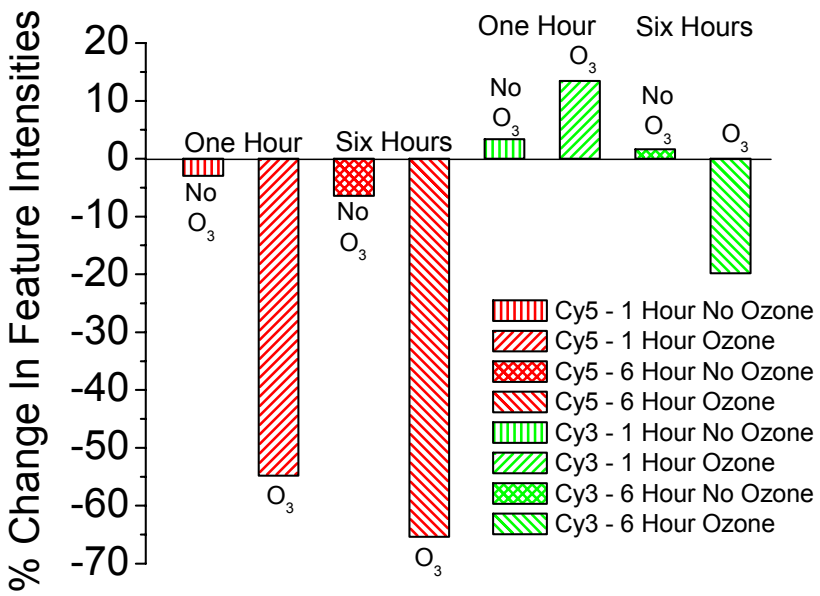

Figure 14. Effect of background ozone on Cy3 and Cy5 channel intensities. Four microarrays were initially scanned immediately after washing. Two of the slides were then moved to an adjacent laboratory in which ozone is not removed by carbon filtration. The remaining two slides were left in the ozone-controlled microarray laboratory. The slides were then rescanned at 1-hr. and 6 hrs. The data are the percent change in both Cy5 and Cy3 feature intensities after 1- and 6-hour exposures to ozone-filtered or nonfiltered room air.

6 hours Cy5 intensity was reduced by 6% in the ozone-reduced laboratory and 65% in the non-ozone-filtered laboratory. Cy3 intensity increased 3% in the ozone-free laboratory and 13% in the nonozone-filtered laboratory after 1-hour. At 6 hours Cy3 intensity was only 2% above initial measurements in the ozone-free laboratory but had fallen to 20% below initial measurements in the non-ozone-filtered laboratory. The effect of ozone is to cause an initial increase followed by a subsequent decrease in Cy3 intensities as has been noted in previous studies (data not shown). Failure to control ozone in the microarray lab will have a large effect on microarray data when Cy5 dye is used. In fact, it may be the biggest cause of variability in microarray experiments because ratio measurements will be dramatically affected by the uncontrolled decay of the Cy5 signal.

Summary

Microarray technology holds great promise in the regulatory arena for drug safety issues. In addition, as biomarkers are discovered, new devices involving this technology are likely to be developed. The FDA has been proactive in moving this technology forward and preparing to accept such data. One of the major hurdles that must be overcome before microarray data can be reliably used in regulatory decision making is the often high degree of variability in gene expression measurements. The microarray procedure is a multi-step process, and subtle technical issues at each stage of the complex method can have major impact on

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the experimental outcome. Here we have discussed issues with microarray fabrication, sample preparation and target labeling and, most importantly, the instability of the commonly used Cy5 dye. It is important for laboratories undertaking microarray studies to recognize and control each of these steps in order to reduce the technical variability so that biologically meaningful information can be extracted. A challenge for the FDA will be assurance that microarray data submitted in support of applications is of high quality.

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Glossary

cDNA — Complementary DNA. It is formed by the conversion of RNA (the gene product) to DNA and is often used as the target for microarray analysis.

Functional genomics — An area of scientific study that aims to discover the biological function of particular genes and to uncover how sets of genes and their products work together in health and disease.

Microarray — A 2-dimensional array, typically on a glass slide or wafer, of genes or gene fragments (often in the form of oligonucleotides) in a predetermined spatial order. The microarray is a powerful technology that allows simultaneous measurement of expression levels for up to tens of thousands of genes.

Oligonucleotide — A short defined sequence of nucleotides (generally fewer than 100 bases). Synthetic oligonucleotides corresponding to portions of a gene are used as probes on microarrays to detect the gene product.

Probe — The oligonucleotide on the microarray that detects a specific gene product.

RNA — The ribonucleic acid molecule that is the direct product of a gene. It directs the synthesis of proteins or is involved in structural or regulatory aspects of cell physiology.

Target — The biological material that is being queried for gene products (RNA). This is usually the RNA from a cell or tissue that has been modified in such a way (e.g., by addition of a fluorescent dye) that its presence can be detected.

Toxicogenomics — A new scientific sub-discipline that combines the emerging technologies of genomics, proteomics, and bioinformatics to identify and characterize mechanisms of action of known and suspected toxicants.

The Authors

James C. Fuscoe is the director of the Center for Functional Genomics, within the Division of Systems Toxicology, at the U.S. Food and Drug Administration's National Center for Toxicological Research (FDA/NCTR). He received his Ph.D. in 1980 from the Graduate School of Biomedical Sciences at the University of Tennessee-Oak Ridge National Laboratory in somatic cell genetics. After postdoctoral research at Baylor College of Medicine on applications of new recombinant DNA methods to the study of mechanisms of mutation in mammalian cells, he joined Lawrence Livermore National Laboratory as a Senior Biomedical Scientist. There he constructed a complete set of human chromosome-specific gene libraries for application to human diseases and helped develop the widely used fluorescence *in situ* hybridization (FISH) technique for mapping and analysis of chromosomal defects. He later joined the faculty of the University of Connecticut, where he was awarded tenure. In 1990, he started working with the U.S. Environmental Protection Agency in developing and utilizing biotechnology in the understanding of mutagenesis and carcinogenesis. In 2001, he joined the NCTR where he established the Center for Functional Genomics to apply gene expression microarray technology to the understanding of toxicology mechanisms and for developing biomarkers. His research interests include application of genomics to toxicology; assessment of age-related susceptibility to chemical toxicants found in foods, drugs, cosmetics, and medical devices; development and application of molecular biomarkers to detect effects of drug exposures; and mechanisms of nongenotoxic carcinogens for application to risk assessment. He was awarded the FDA Commissioner's Special Citation Award in 2005 for his work in pharmacogenomics.

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1996. She moved to Arkansas in 1999 and continued as a microbiologist and later as a supervisory microbiologist at the Arkansas Regional Laboratory. In 2002, she began working at NCTR in the Center for Functional Genomics where she utilizes microarray technology for the understanding of toxicological mechanisms.

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Carrie L. Moland is a biological laboratory technician, working with the Systems Toxicology Division, specializing in gene microarray at the National Center for Toxicological Research (NCTR). She received an associate degree from Southeast Arkansas College and a diploma in computer programming from South Centre Career College. Ms. Moland joined NCTR in 1986 as a pathology contractor and, in 1996, started working for the Food and Drug Administration. During her years in pathology, she set-up and managed a necropsy and histology laboratory. She has expertise in necropsy (all types of animal work), histology, and teratology, in addition to her skills in RNA isolation and microarray hybridizations.

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Weida Tong received his Ph.D. in Chemistry in 1990. Following his appointment as a research associate in computational chemistry at the University of Missouri-St. Louis, he joined the FDA's NCTR as a contractor focusing on development of QSAR models for hormone binding and endocrine disruption, and bioinformatics approaches for genomics and proteomics. In June 2002, he became the Director of NCTR's Center for Toxicoinformatics with the responsibility of leading a multidisciplinary team in developing ArrayTrack and other bioinformatics and chemoinformatics software and methods in support of "omics" research in FDA. He is also an adjunct associate professor in the Department of Pharmacology, Robert Wood Johnson Medical School of the University of Medicine & Dentistry of New Jersey (UMDNJ), as well as assistant professor in the Department of Pharmaceutical Sciences at the University of Arkansas for Medical Sciences. Dr. Tong has published about 80 papers and book chapters in

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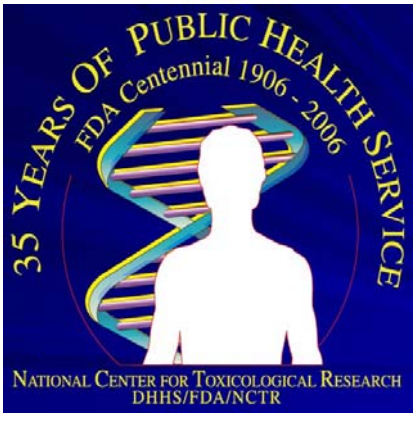
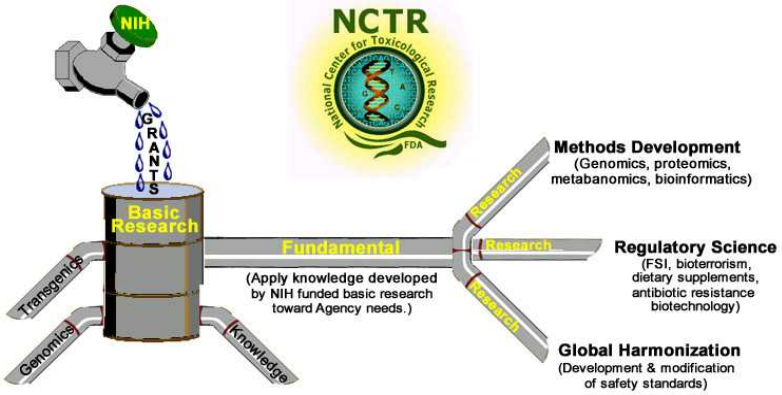
computational toxicology, computational biology, and bioinformatics.

Adam T. Scully is a licensed professional engineer and a project manager for design, construction, and renovation projects at the NCTR, Jefferson Laboratories, at Jefferson, Ark. Mr. Scully received a B.S. in mechanical engineering from the Virginia Polytechnic Institute and State University in 1985 and has worked at Westinghouse, Naval Surface Warfare Center, the National Institutes of Health, and Indian Health Services, before joining the Food and Drug Administration in 2003. In addition to coordinating construction program activities, Mr. Scully has received numerous awards for his engineering efforts to save on construction and energy costs.

Robert R. Delongchamp received an MPH from the University of Michigan in 1973. After a year as a fishery statistician with the Ecological Services group of Texas Instruments, he joined the NCTR's Biometry Division as a mathematical statistician, where he served as a statistical consultant and collaborator for the next ten years. In 1984, he left NCTR to pursue a doctorate in Statistics at Oregon State University. In 1988, he accepted a position with Dow Corning Corporation to evaluate the existing human and animal data relevant to the health effects of silicone breast implants. He completed his Ph.D. in 1993 and then spent three years at the Radiation Effects Research Institute in Japan, where he was responsible for the analysis of the follow-up study of *in utero*-exposed survivors to the atomic bombings. Since returning to NCTR's Division of Biometry and Risk Assessment in 1996, Dr. Delongchamp has conducted research toward the development of statistical methods for mutation assays that use transgenic animals, statistical methods to estimate genotype frequencies from a subject's phenotype, and statistical methods to evaluate cDNA array data for altered gene expression. A theme throughout Dr. Delongchamp's career has been to provide a sound scientific interpretation of toxicological or epidemiological data that underlie risk assessment.



The NCTR research integrates traditional toxicology and innovative new technologies to develop a toolkit for translating basic research from others and to apply it to public health needs.



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