Effects of Atmospheric Ozone on Microarray Data Quality

Thomas L. Fare, Ernest M. Coffey, Hongyue Dai, Yudong D. He, Deborah A. Kessler, Kristopher A. Kilian,* John E. Koch, Eric LeProust,† Matthew J. Marton, Michael R. Meyer, Roland B. Stoughton, George Y. Tokiwa, and Yanqun Wang

Rosetta Inpharmatics LLC, 12040 115th Avenue NE, Kirkland, Washington 98034, and Agilent Technologies, 3500 Deer Creek Road, Palo Alto, California 94304

A data anomaly was observed that affected the uniformity and reproducibility of fluorescent signal across DNA microarrays. Results from experimental sets designed to identify potential causes (from microarray production to array scanning) indicated that the anomaly was linked to a batch process; further work allowed us to localize the effect to the posthybridization array stringency washes. Ozone levels were monitored and highly correlated with the batch effect. Controlled exposures of microarrays to ozone confirmed this factor as the root cause, and we present data that show susceptibility of a class of cyanine dyes (e.g., Cy5, Alexa 647) to ozone levels as low as 5−10 ppb for periods as short as 10−30 s. Other cyanine dyes (e.g., Cy3, Alexa 555) were not significantly affected until higher ozone levels (>100 ppb). To address this environmental effect, laboratory ozone levels should be kept below 2 ppb (e.g., with filters in HVAC) to achieve high quality microarray data.

Reports in the literature have noted seasonal effects on microarray data quality.[1] The intermittent nature of these effects makes tracking down the root cause difficult and time-consuming. During a series of experiments, we observed that fluorescent signal intensity decreased and a ratio reproducibility parameter (defined in Methods Section below) consistently failed its passing threshold value. Closer examination of the arrays showed two effects: First, there was an array-wide gradient for signal intensity and ratios. Second, individual features had reduced Cy5 signal intensity relative to Cy3 intensity with a spatial intensity profile characteristic of the anomaly (see comparison in Figure 1 of spots with expected morphology versus spots that display the anomaly, namely, a green ring and inscribed red circle).

In the course of data analysis, an observation was made that data quality of batch-processed arrays might be correlated with environmental ozone levels during posthybridization array washing. To follow up on this lead, we designed two experiments to determine the point at which the effect would be induced. A first set of experiments was to determine if the effect was localized to posthybridization processing; the second set was designed to evaluate specifically the contribution of wash protocols on the effect. In this work, we describe a series of experiments that identified the root cause and the actions taken to address the source of the effect.

METHODS SECTION

Microarray experiments were performed using a two-color, fluor-reversed pair platform, as previously described.[2] Briefly, RNAs were amplified by standard methods and hybridized to Agilent microarrays (Part G2509A, Palo Alto, CA) in custom hybridization cartridges. The posthybridization wash step is described in detail below. All arrays were scanned on the Agilent microarray scanner (model G2565AA, Palo Alto, CA). Image processing and feature extraction and quantitation were performed as described.[3] Microarray data quality was assessed using a quality control template of spatially addressable probes designed to assess microarray synthesis and assay sensitivity and specificity. This subset of probes is common to every microarray pattern, allowing one to calculate quality parameters.

Posthybridization Wash Protocol. In the standard Rosetta wash protocol, slides are removed from hybridization cartridges and immediately submerged in a 50-mL conical tube containing a primary wash solution (6× SSPE, 0.005% sarkosyl), inverted 20 times, quickly transferred to a new tube containing a secondary wash solution (0.06× SSPE), inverted 20 times, and then removed from the tube and placed into a slide box to dry. For the set of experiments described below, drying was performed either in the presence of controlled amounts of ozone or under positive pressure of nitrogen gas.

Exposure of Microarrays to Controlled Ozone Levels. To create controlled ozone levels for array washing experiments, we set up an enclosed chamber (PLAS LABS, Lansing, MI) with an ozone generator (Multizone 200 H.O. Ozone Generator, Crystal Air Canada, Inc.) placed inside. Ozone levels were monitored using a UV absorption ozone analyzer, model 400A (Teledyne ABI, San Diego, CA), and the monitor was hooked up to the chamber to provide feedback to control ozone levels during exposure. Prior to each experiment, primary and secondary wash solutions, falcon tubes, waste container, tweezers, and slide boxes (depending on the number of arrays per experiment) were brought into the chamber.

---

* Corresponding author. E-mail: kristopher_kilian@merck.com.
† Agilent Technologies.

(1) http://www.genisphere.com/array_detection_troubleshooting_res.html #19, http://www.genisphere.com/pdf/DyeSaverPoster.pdf.

(2) Hughes, T. R.; Mao, M.; Jones, A. R.; Burchard, J.; Marton, M. J.; Shannon, K. W.; Lefkowitz, S. M.; Ziman, M.; Schelter, J. M.; Meyer, M. R.; Kobayashi, S.; Davis, C.; Dai, H.; He, Y. D.; Stephaniants, S. B.; Cavet, G.; Walter, W. L.; West, A.; Coffey, E.; Shoemaker, D. D.; Stoughton, R.; Blanchard, A. P.; Friend, S. H.; Linsley, P. S. *Nat. Biotechnol.* **2001**, *19*, 342.

(3) Marton, M. J.; DeRisi, J. L.; Bennett, H. A.; Iyer, V. R.; Meyer, M. R.; Roberts, C. J.; Stoughton, R.; Burchard, J.; Slade, D.; Dai, H.; Basset, D. E. Jr.; Hartwell, L. H.; Brown, P. O.; Friend, S. H. *Nat. Med.* **1998**, *11*, 1293.
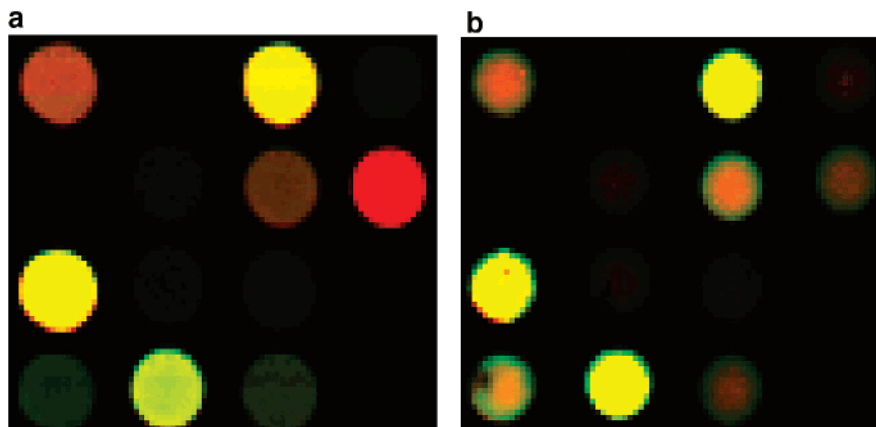
Figure 1. (a) Image of a microarray section displaying high-quality features and (b) the same section of a different microarray with the intraspot anomaly that occurs with the ratio reproducibility failure.

Exposure of Artifact Free Microarrays to Ozone during the Washing and Drying Steps. Eight previously hybridized arrays known to be free of the defect were prescanned immediately prior to this experiment to confirm they still retained good signal quality. Wash solutions were poured into Falcon tubes inside the ozone chamber, and then two arrays at a time were brought into the chamber in a closed, plastic microscope slide box. Arrays were taken out of the slide box one at a time, washed in primary and secondary wash solutions, respectively, in the ozone environment and then placed in a second slide box opened to the ozone environment for ~1 min. Following ozone exposure, the slides were immediately scanned.

Modification of Wash Procedure To Assess Effect of Ozone during Drying. In this round, 48 arrays were hybridized specifically for ozone exposure during the washing and drying steps. The wash procedure was modified such that arrays were kept submerged under buffers to the extent possible during wash steps in the ozone chamber to determine if wash solutions absorb significant ozone amounts to contribute to the effect. Arrays were washed and dried inside the chamber either in 12.5 ($\pm$ 2.5) ppb ozone ambient or with a positive pressure of dry nitrogen to minimize the effect of ozone.

Ratio Reproducibility Quality Metric. Two parameters were measured to determine the effect of ozone on the arrays: fluorescent intensity and ratio reproducibility. Fluorescent intensity averaged over all features is corrected for background and given in arbitrary units. Ratio reproducibility is a quality metric that determines how consistently the Cy3/Cy5 signal ratio of known synthetic transcript spike-ins can be recovered; the metric is measured using synthetic transcripts added to total RNA at known concentrations (prior to amplification and labeling).[2] Microarray probes complimentary to spike-in sequences are distributed across the array and are evaluated to determine whether the same ratio can be recovered across the array. The metric is defined as

Ratio reproducibility =

$$\text{Max}_k \left(\sum_{i=1}^{N_k} \left(\log_{10} \frac{R_k(i)}{G_k(i)} - \frac{\sum_{i=1}^{N_k} \log_{10} \frac{R_k(i)}{G_k(i)}}{N_k} \right)^2 \middle/ (N_k - 1) \right)^{1/2}$$

where k is the number of spike-ins examined, N_k is the number of repeats for the kth spike-in on the chip, and $R_k(i)$ and $G_k(i)$ are the normalized red and green channel intensities, respectively, for the ith repeat of the kth spike-in. This reproducibility measure is, in essence, the maximum of the standard deviation of the k spike-in ratios. It is reasonable to expect that the lower the abundance a spike-in sample is, the larger its standard deviation will be. To avoid the effect of background noise when the transcript abundance is too low, five spike-ins of adequate abundance are chosen so that the abundance is 10 copies/cell in one channel and 10, 30, or 100 copies/cell in the other channel, with the $\log_{10}$ (ratio) being -1, -0.5, 0, 0.5, and 1. The five spike-ins are fixed in our process so that reproducibility can be compared across slides. The threshold for passing/failing the ratio reproducibility is set at 0.06 subjectively on the basis of normalization of historical data.

RESULTS

Exposure of Artifact-Free Microarrays to Ozone during the Washing and Drying Steps. Ratio reproducibility data for exposure of eight arrays to ozone that had previously passed the ratio reproducibility metric are shown in Figure 2. Data in the left panel (first scan) show that these arrays passed the metric immediately prior to exposure (within the hour). Arrays were then washed in the ozone chamber, two at a time, then dried in the chamber. Different array sets were exposed to a range of ozone concentrations for ~60 s. In the right panel, we show that the ratio reproducibility metric increases on array pairs after exposure and the metric worsens at higher ozone exposure level. At a 20 ppb exposure for 1 min, the arrays have already crossed the metric failing threshold (indicated by the blue line in the plot). Rewash controls run at very low ozone (<5ppb) show that simply rewashing the slides does not significantly contribute to the observed effect (see arrays 9 and 10 in Figure 2).

In addition, this ozone exposure induced the intraspot anomaly characteristic of this effect (see Figure 3, taken from slide 16011011014779 in Figure 2). The inset image (Agilent Technologies, Image Analysis Version A.4.0.34, Palo Alto, CA) shows a section of an ozone-exposed array containing the anomaly. We show a plot of Cy5 (red) and Cy3 (green) signal intensity traced through the spots designated by the red arrow. We noted that a given spot has relatively uniform green intensity, whereas the overall red intensity has decreased (relative to its initial value),
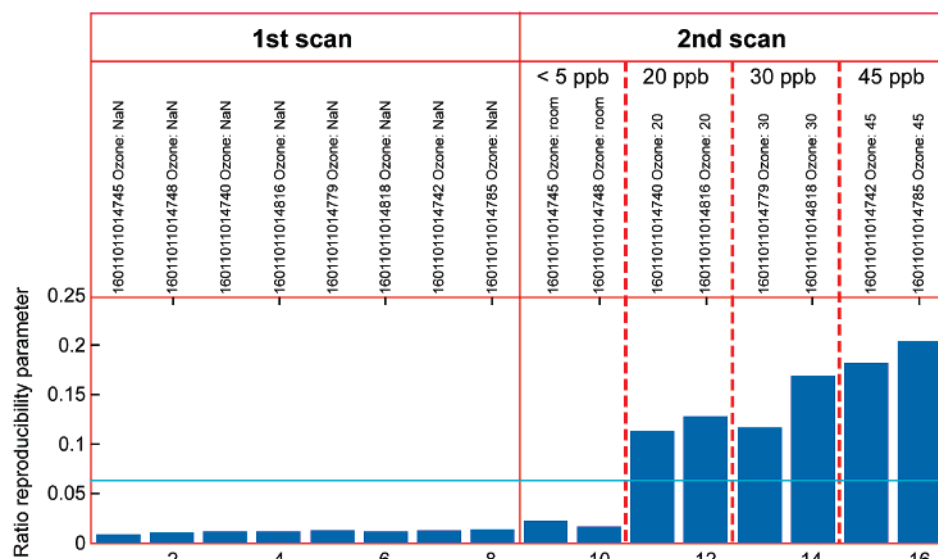
Figure 2. Results from array ozone exposure during wash steps. A larger value in the *Y* axis indicates worse ratio reproducibility. Total time of ozone exposure for a given pair was 1 min. Data in the left panel are for arrays that had previously passed and were rescanned just prior to exposure; the right panel shows results from the same arrays after exposure to indicated ozone levels (note barcodes). The horizontal blue line is the pass/fail quality threshold.
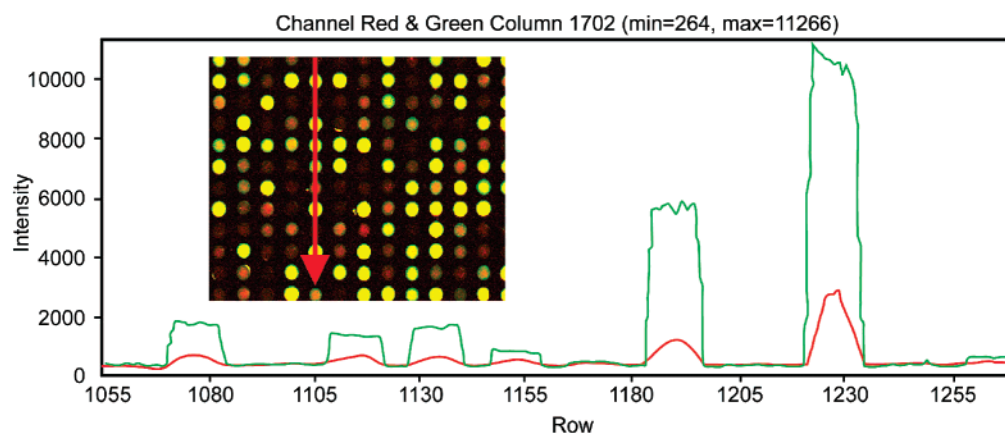

Figure 3. Image (inset) and fluorescence intensity trace through spots (slide 16011011014779 in Figure 2) designated by red arrow shows the induced ozone effect is identical to the data anomaly in failed arrays.

with a peak in the center of the spot, and symmetrically tails off toward the spot edge. The combination of uniform green intensity with the profile of the red intensity creates the characteristic appearance for the defect.

Modification of Wash Procedure To Assess Effect of Ozone during Drying. In Figure 4, we show results from tests to determine whether ozone induces this effect either during washing or during drying. In this experiment, 48 arrays were all hybridized simultaneously, then split into two groups of 24, each group to be washed by two separate treatments. Within each group, one-half of the arrays were immediately set in the nitrogen purge box after being removed from the second wash buffer, while the remaining arrays were left open to dry in the environment. In wash treatment 1, 24 arrays were transferred between hybridization and wash buffers under water but in an overpressure of 12.5 (± 2.5) ppb ozone, then dried under either nitrogen or 12.5 (± 2.5) ppb ozone. In wash treatment 2, 24 arrays were transferred between hybridization and wash buffers through the 12.5 (±2.5) ppb ozone overpressure, then dried under either nitrogen or 12.5 (±2.5) ppb ozone. As can be seen in the figure, the condition that

induced the effect was the drying condition. Hence, we can discount ozone absorbed into the wash solutions and ozone exposure during transfer to wash as contributing to the effect; furthermore, we speculate that as long as water could provide a diffusion barrier to ozone during the drying step, the dyes would be protected.

Effects of ozone on dry arrays after hybridization were also studied. In these cases, we exposed arrays to ozone at levels ranging from 60 to 85 ppb for periods of 5, 10, 20, and 30 min. Cy3 dye essentially retained its intensity level (compared before and after exposure); Cy5 dye started to lose intensity after ~10 min, but then remained relatively flat thereafter at about one-third the preexposure intensity level (data not shown). Intensity loss for these exposures tended to be uniform across a feature, rather than resulting in the characteristic defect shape caused by ozone exposure during the drying process.

We also noted similar effects at low ozone levels for another cyanine dye, Alexa 647, which has peak fluorescences at a wavelength equivalent to Cy5. Furthermore, we observed that Cy3 and Alexa 555 did have somewhat reduced signal at low-ozone exposures, but was within experimental tolerance. Effects on Cy3
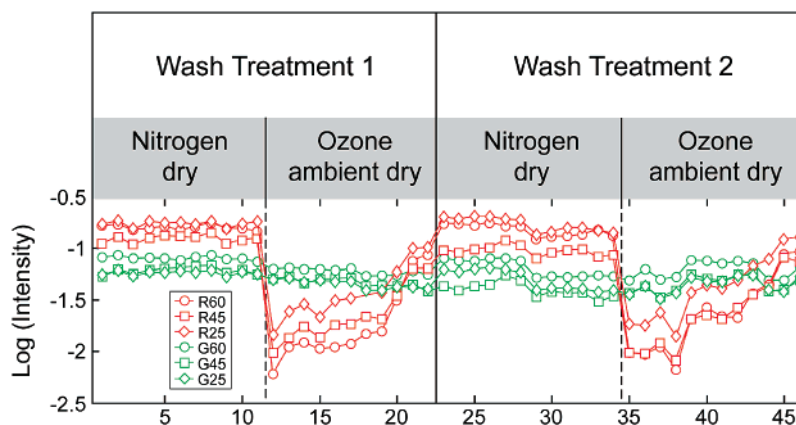
Figure 4. Comparison of the log intensity of two wash treatments (see text for details) under two drying conditions: dry nitrogen and 10−15 ppb ozone. For the two drying conditions on the left, arrays were transferred under buffers throughout processing to avoid ozone exposure. For the two drying conditions on the right, arrays were washed with normal (air) transfer from hybridization buffer to wash buffer 1 to wash buffer 2. Arrays in the figure are presented in order of processing (within treatment). Total time of exposure from first to last array within treatment was ∼30 min; note that the lowest intensities are observed for those arrays that have been exposed to ozone the longest. R60, R45, and R25 are defined as the Cy5 intensity of the 60-mer, 45-mer, and 25-mer oligonucleotide probes, respectively. Likewise, G60, G45, and G25 are defined as the Cy3 intensity of the 60-mer, 45-mer, and 25-mer probes.

or Alexa 555 become significant at ozone levels greater than ∼100 ppb for exposure times on the order of minutes (data not shown).

CONCLUSIONS

On the basis of the above data, we conclude that environmental ozone exposure after the secondary wash step was the root cause of the data anomaly. One of the significant findings of this work was the low dose level (ozone concentration multiplied by exposure time) that could induce the onset of the phenomenon, suggesting many labs may be at risk. For example, we measured environmental ozone levels outside our laboratory that would exceed 60 ppb during peak traffic hours on a sunny summer afternoon. Fluorophores on arrays exposed to these levels for as short as 1 min will begin to show significant degradation in typical laboratory settings. In fact, reports of this effect in discussion groups[1] on other microarray platforms suggest it may manifest itself differently or occur at different stages of the process, depending on the process/platform and the ozone level.

We propose that the physical characteristics of the anomaly (array-level gradient, intraspot defect feature) could be caused by the drying pattern of the wash buffer on the DNA spots. Because the surface of the array outside the spots is hydrophobic on Agilent arrays, wash buffers sheet off the slide, leaving the DNA spots wetted with beads of buffer. As long as buffer remains on a spot, it would afford protection as a diffusion barrier against ozone exposure. In general, we propose that the wetted spots tend to dry from the edge of the slide inward, thus exposing edge spots first to create the array-level gradient. Buffer on the DNA spots dries from the circumference of the spot inward, thus exposing the outer ring of the spot first to create the characteristic defect

feature. Combined with the fact that completely dry arrays have Cy5 channel intensity depleted much slower than the arrays during the drying process, it's very likely that the Cy5 depletion is faster during the wet/dry phase transitions.[4−6]

To reduce effects of ozone on microarray data, we recommend several effective actions: first, when possible, microarray work should be performed in a laboratory with an HVAC system outfitted with filters that significantly reduce ozone (≤2 ppb) and second, arrays should be stored in a positive-pressure nitrogen box after washing. By implementing both of these changes, we were able to completely eliminate the occurrence of the ozone effects on our microarray data. Alternatively, a dye-protecting solution available from Agilent Technologies has been demonstrated to prevent both the Cy5 signal degradation and the associated loss of data quality at elevated ozone concentrations.[7] We were able to rule out other contributors to ozone generation, such as internal instruments and the HVAC system itself; however, electrical motors can create substantial ozone within a confined area and should be kept out of microarray laboratories. Further investigation into environmental effects during processing with other microarray platforms is recommended.

Note Added after ASAP: This paper was posted on the Web before all corrections had been made. The paper was reposted on 7/25/03.

(4) *Ozone Chemistry and Technology, A Review of the Literature 1961−1974*; The Franklin Institute Press: Philadelphia, 1975.
(5) Valdes-Aguilera, O.; Cincotta, L.; Foley, J.; Kochevar, I. E. *Photochem. Photobiol.* **1987**, *45*, 337.
(6) Hori H.; Nakagawa, Y.; Ojima, H.; Niijima, T.; Terada, H. *Adv. Exp. Med. Biol.* **1992**, *317*, 255.
(7) Agilent application note 5988-9666EN , Drying and Protective Solution; Agilent part no. 5185-5979; obtainable from http://www.chem.agilent.com/scripts/Library.asp.