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Method to minimize ozone effect on Cy5 fluorescent intensity in DNA microarrays

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ABSTRACT

Cyanine 5 (Cy5) is an established fluorescent dye in microarray analysis. It is degraded rapidly when exposed to atmospheric ozone during post-hybridization washes, which leads to loss of fluorescent intensity. To minimize this undesirable effect, we coated microarray slides with sodium dodecyl sulfate (SDS) solution at post-hybridization washes. The fluorescent intensities on coated slides were more stable than those on uncoated slide. We also performed the microarrays with SDS solution for a year to check the solution's effectiveness along with seasonal changes of atmospheric ozone level. Consistent results in microarray analysis were obtained using Cy5 dye under atmospheric ozone.

Introduction

Cyanine dyes (Cy3, Cy5) are fluorescent dyes used in microarray analysis. Cy5 molecules are detrimentally affected by environmental ozone, which degrades the molecules during washing and drying steps in microarray analysis [1,2]. Several methods have sought to avoid this undesirable effect; these include eliminating ozone by installing carbon filter in the laboratory setting [3], substitution of Cy5 by another fluorescent dye that displays stable fluorescent intensity at high ozone levels [4] and using commercial buffers to treat the microarray slides [5]. However, the different fluorescent dyes and commercial solutions can be expensive and can have procedural complications.

In this research, we describe a simple way to reduce the ozone effect on the Cy5 during DNA microarray analysis. Coating the microarray slides with sodium dodecyl sulfate (SDS) solution after hybridization washing was effective in maintaining the fluorescent intensities of the dyes. We also investigated the effect on Cy5 fluorescent dyes of seasonal variations in atmospheric ozone. All the microarray experiments were performed using human papillomavirus (HPV) DNA chip [7].

Materials and methods

Measuring atmospheric ozone level

The data of atmospheric ozone level was from the website of Seoul air quality information (http://cleanair.seoul.go.kr) that provides the concentration of ultrafine particles, nitrogen dioxide, carbon monoxide, and ozone in the administrative district of Seoul.

Microarray

The Cheil HPV DNA chip was used for this analysis according to the protocols described previously [7]. This microarray for genotyping HPV was constructed using oligonucleotide probes set designed specifically for a part of HPV L1 structural gene (HPV 6, 11, 16, 18, 30, 31, 32, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 62, 66, 67, 68a, 68b, 69, 70, 72, 81, 82, 84, 90, 91), internal control (human beta globin) and position marker. These

Abbreviations: Cy5, Cyanine 5; Cy3, Cyanine 3; SDS, sodium dodecyl sulfate; Alt, alternative dye; HPV, human papillomavirus; PCR, polymerase chain reaction; ppb, part per billion.

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oligonucleotides were dissolved in spotting buffer at each concentration of 25 μM and arranged on aldehyde-coated glass slide using an OmniGrid Accent microarrayer (DIGILAB, USA) and immobilized. This oligonucleotides set was printed identically at eight different locations on the slide. After immobilization, the slides were incubated with sodium borohydride solution containing 0.3% $NaBH_4$, 25% ethanol with phosphate-buffered saline buffer for 20 min to reduce the imine group of its surface. The slides were stored in a desiccator at room temperature. This microarray was approved by Korea Food and Drug Administration. The 36 types of HPV L1 gene were cloned into DNA plasmids as a target [7]. The specific region of L1 gene of the plasmid was amplified by real-time PCR using L1 consensus primers labeled with cyanine dyes (Cy5, Cy3, BIONEER, Korea) or ATTO647 (ozone resistance dye, ATTO-tech, Germany). Each fluorescent dye labeled PCR products were hybridized with probes on the slide. Detailed procedures of real-time PCR and hybridization were also described previously [7].

Scanning and analysis

The hybridized slides were scanned using a NimbelGen MS 200 device (Roche, Basel, Switzerland) to obtain images using the 532 nm and 635 nm channels. The images from the scanner were analyzed using Genepix 5.0 software, measuring the fluorescent intensities of all spots on the slide. For the fluorescent signal value, the median value of all pixels within a spot was used for analysis. The ratios of fluorescent signal were calculated that the mean of Cy5 or Alt fluorescent signal values within all the spots on the slide was divided by Cy3 fluorescent signal values within same slide.

Statistical analysis

Wilcoxon signed-rank test was employed using SPSS Statistics 12 (SPSS, Inc., Chicago, IL, USA) to analyze whether the difference between two fluorescent signals at each scanning were statistically significant at 5% significant level.

Results and discussion

To minimize the negative influence of ozone on Cy5 molecules in microarray analysis, we coated microarray slides with SDS after post-hybridization washing. The templates (HPV type 16 and 18 cloned into plasmids) were amplified and labeled by Cy5, Cy3 and Alt (ATTO647, alternative dye of Cy5) using real-time PCR. The five products (Cy5-product, Cy3-product, Alt-product, Cy5-product mixed with Cy3-product, and Alt-product mixed with Cy3-product) were each prepared and hybridized in five wells on the slide. The two microarray slides were hybridized identically. After post-hybridization washing, one of the slides was submerged in a 0.05% SDS solution for 30 s and the other remained in the final washing buffer. Both slides were dried and scanned. After the first scanning, the slides were placed in the laboratory where the ozone was uncontrolled and exposed to the air for 20 min and scanned again. This procedure was repeated six times.

The fluorescent intensities were compared between two fluorescent dyes (Cy5 and Alt) as well as between two slides (SDS treated and untreated) (Fig. 1). At the final scan, Cy5 fluorescent intensities were not changed significantly in wells of SDS-treated slides. However, lack of SDS treatment was associated with markedly reduced fluorescent intensities. The Alt fluorescent intensities were not changed. Reduced fluorescent intensity was apparent at the fourth scan when the atmospheric ozone level was elevated about 2-fold from the previous level. Similarly, the fluorescent intensities of internal control (human beta globin) labeled only by Cy5 were also reduced on the slides that not coated by SDS solution, but were maintained on slides treated with SDS solution. Examination of the fluorescent intensities including position markers (immobilized probes) showed that the SDS solution did not amplify fluorescent intensities.

The Cy5/Cy3 and Alt/Cy3 ratios clearly show the changes of the fluorescent intensity for scanning (Table S1). At the first scan, the ratios were more than 1.00. The Cy5/Cy3 ratio became lower with repeated scans while the ratio of Alt/Cy3 was not reduced. Although the Cy5/Cy3 ratio was reduced on both slides, SDS treatment resulted in a more gradual decrease of the ratio compared to untreated slides.

To test whether the differences of each fluorescent signal with repeated scans were statistically significant compared to the first scan, the Wilcoxon signed-rank test was performed (Table S1). The fluorescent signals in all wells on the slide were included for the analysis. On untreated slides, the difference of Cy5 signals were significant beginning with the fourth scan ($p = 0.0002$), while all other signals were not significantly different. Moreover, at the last scan compared to the first scan, the remaining Cy5 fluorescent intensity was only 2% on untreated slides, but 70% of the fluorescent intensity remained on the SDS-treated slides.

We applied SDS to our routine DNA microarray every 4 weeks for a year to investigate the effect of seasonal atmospheric ozone level. Each microarray was performed when the mean ozone level based on Seoul Air Quality information was the highest during the day (10:00–17:00). The Cy5 fluorescent signals tended to decrease when the ozone level was relatively high (Fig. 2), and the Cy5/Cy3 ratio also shows similar tendency in untreated slides (Fig. S1). However, SDS-treated slides showed more stable Cy5 fluorescent signals compared with untreated slides. This suggested that the Cy5 fluorescent signals were affected by atmospheric ozone on our microarray, in agreement with previous reports that the ozone affects Cy5 fluorescent signal [1–4], and that the SDS solution is effective in maintaining Cy5 fluorescent signals on microarray slides.

The present method is a simpler, filter-free way to reduce the negative effect of ozone on Cy5 in microarray analysis. Like the commercially available stabilization and drying solution [5], in our method the SDS solution coated the slides to provide a barrier to atmospheric ozone (Fig. 1). The Cy5 fluorescent intensities were maintained in the SDS treated condition. Moreover, we did not see increasing fluorescent intensities including Cy3 and Alt when the SDS solution treated (Fig. 1). This suggests that the solution did not merely amplify the fluorescent intensities.

To check the seasonal changes of the Cy5 fluorescent intensity affected by atmospheric ozone, we performed the microarray analysis once a month for a year. Cy5 fluorescent intensities are affected by humidity [6]. In May to August, the humidity was relatively high compared with other months. However, the microarrays were performed in the laboratory where humidity, but not ozone, was controlled. In addition, to offset the other unknown factors, we determined the Cy5/Cy3 ratio in microarray analysis (Fig. S1). The Cy5/Cy3 ratio was inversely associated with the atmospheric ozone level in the absence of SDS.

To clarify the effects of SDS on Cy5 dye, we measured the fluorescent intensity using different concentrations of SDS coating solution: 0%, 0.01%, 0.05%, and 0.1% at 63 ppb atmospheric ozone concentration (Fig. S2). The Cy5 fluorescent intensities were blurred in the 0% and 0.01% SDS solution groups, but no blurring was observed when solutions of over 0.05% concentration were used. Analysis of the fluorescent signal values at each SDS concentration (Table S2) showed that the Cy5 values were considerably lower at concentrations of 0% and 0.01% than at 0.05% and 0.1%. In addition, the Cy5 fluorescent signal values tended to increase with SDS concentration, whereas those of Cy3 showed no clear trend.