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Nondestructive Quality Control for Microarray Production

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ABSTRACT

The use of microarrays to monitor gene expression has become a standard research tool at both academic and industrial research institutions. Quality control of common printing defects during DNA deposition onto glass substrates is critical to maintaining data integrity and preventing the need-

less consumption of precious RNA, labeling reagents, and time. Here we demonstrate a nondestructive method for monitoring the quality of every spot on every chip of a microarray production run. We have identified many common manufacturing defects, while not perturbing the attachment of our oligonucleotide target to the substrate or altering further hybridization. This protocol is simple, fast, and inexpensive.

INTRODUCTION

The use of microarrays to monitor gene expression has become a standard tool at large academic and industrial research institutions (6). The microarray process flow is highly variable and still evolving. Quality control of common printing defects during DNA deposition onto glass substrates is critical to maintaining data integrity and preventing the useless consumption of precious RNA, labeling reagents, and time (15). Common size defects include spot coalescence, small spots, and missing spots. Spot morphology defects are also common. These include "ringing" or "donuting", noncircular spots, and other shape defects caused by debris collected on spotting pins. Several methods have been used successfully to control for these printing defects. When cDNA target is PCR-amplified with biotinylated primers, fluorophore-coupled streptavidin can be used to stain the array before hybridization. Hybridization of an array with a labeled oligonucleotide corresponding to a polylinker sequence present in all of the spotted PCR products has been used successfully (3). Similarly, Cy[®]3- and Cy[®]5-labeled random 9-mers can be hybridized to assess spot quality. Other quick and effective staining techniques utilize ethidium bromide, OliGreen[®], SYBR[®] Green II, and SYTO[®] 61 dyes (Molecular Probes, Eugene, OR, USA) (1,18). The above techniques require a time-consuming incubation step. In addition, the assayed slide shows reduced performance or is not available for subsequent hybridization with labeled sample probe. Therefore, only a few slides can be quality tested, perhaps the first and last slide in a print run. The data are then extrapolated across the entire production run, thereby missing

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possible defects in the remaining slides. New automation technologies can provide an alternative to these staining techniques by monitoring spot quality during manufacturing, but this only allows for verification of deposition and does not control for morphology of the spot following hybridization. Food coloring has also been used as a means to control for uniform and consistent spots when optimizing chip manufacturing parameters (10). Here we demonstrate a nondestructive method for monitoring the quality of every spot on every chip of a microarray production run. We have identified the morphology and size defects listed above, while not perturbing the attachment of our oligonucleotide target to the substrate or altering the hybridization intensity.

MATERIALS AND METHODS

Array Fabrication

5' amine modified 30-, 50-, or 70-mer oligonucleotides with a C-12 linker were synthesized and purified by MWG Biotech (High Point, NC, USA). Oligonucleotides were diluted to a final concentration of 20 pmol/ μ L in 150 mM NaPO₄ buffer at pH 8.5 with 0.001% SDS. Various concentrations of dCTP-Cy3 or dCTP-Cy5 (Amersham Biosciences, Piscataway, NJ, USA) were spiked into each source plate sample well. A MicroGrid™ TAS II with 16 Microspot™ 2500 quill pins (Biorobotics, Woburn, MA, USA) was used to array oligonucleotides at various pitches to Surmodics slides (Eden Prairie, MN, USA). Oligonucleotides were arrayed from 20 μ L sample in 384-well V-bottom plates (Genetix, Hampshire, UK) at 30% humidity. Resulting spot sizes averaged 140 μ m.

Pre-Hybridization Processing

Slides were processed according to Surmodics recommended protocols. In brief, attachment of the oligonucleotides to substrate was achieved by incubation for 16 h in a saturated NaCl chamber. Slides were stored at room temperature in a vacuum desiccator until further use. Before hybridization, slides were incubated for 20 min at

50°C in 50 mM ethanol amine, 0.1 M Tris, 0.1% SDS, pH 9.0. Slides were rinsed twice with deionized-distilled water at room temperature and then washed for 40 min with 4 $\times$ saline sodium citrate (SSC), 0.1% SDS prewarmed to 50°C. Slides were washed twice in room temperature deionized-distilled water using gentle shaking, centrifuged dry at 100 $\times$ *g* for 3 min, and immediately hybridized.

RNA Labeling

II-23 T-cell hybridoma cells were induced with 280 ng/mL calcium ionophore ionomycin and 25 ng/mL phorbol ester PMA. RNA was harvested from wild-type and induced cells by TRIZOL® (Invitrogen, Carlsbad, CA, USA) extraction 6 h after induction. Extracted RNA was cleaned using RNeasy® kit (Qiagen, Valencia, CA,

Figure 1. Concentration titration of dCTP-Cy3. 30-mer oligonucleotides were spiked with dCTP-Cy3 at various concentrations and scanned. The Cy dye spike was either absent from (D) or present at concentrations of 10 (A), 1 (B), or 0.1 pmol/ μ L (C). (E–H) Slides were washed according to the prehybridization protocol and scanned once again.

Figure 2. Hybridization of oligonucleotide targets containing dCTP-Cy3 spikes. A sense (middle and bottom rows) and antisense (top row) 30-mer oligonucleotide target was arrayed and hybridized using an equal molar amount of Cy3 and Cy5 end-labeled 30-mer oligonucleotide probe complementary to the sense target. Oligonucleotide targets were either arrayed without quality control spike (bottom row) or with 0.5 pmol/ μ L dCTP-Cy3 (top and middle rows). (A) Array before prehybridization washing. (B) Panel A following washing. (C) Panel B following hybridization.

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USA). Fluorescently labeled cDNA was generated by standard protocols using 20 µg total RNA, dCTP-Cy3 and dCTP-Cy5 dyes, and SUPERSCRIPT II® reverse transcriptase (Invitrogen) (8). Probe was quantified at 260 and 550 nm for Cy3 labeling or 650 nm for Cy5 labeling using a Beckman DU640B spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The amount of incorporated dye was calculated for each labeling reaction.

Hybridization and Washing

5' Cy-labeled 28-mer probes were manufactured and HPLC-purified by Oligo's Etc. (Wilsonville, OR, USA). These five unique probes were complementary to target 30-mers on the glass substrate. The hybridization cocktail consisted of 22.8 fmol each probe labeled in both the Cy3 and Cy5 channels, 5× SSC, 0.1% SDS, and 0.1 mg/mL salmon sperm DNA (Invitrogen) in a total volume of 22 µL. The oligonucleotide mixture was hybridized under a 22 × 40 mm glass coverslip for 16 h in a 42°C water bath using a hybridization chamber (Corning, Corning, NY, USA). Following hybridization, slides were washed twice at room temperature in 2× SSC, 0.1% SDS for 5 min, 0.2× SSC for 5 min, and finally 0.1× SSC for 5 min. The slides were dried by centrifugation.

Cy3- and Cy5-labeled cDNA probes were combined based on 10 pmol incorporated dye each and then dried and resuspended in 15 µL hybridization solution consisting of 5× SSC, 0.1% SDS, and 0.1 µg/mL salmon sperm DNA. The probe was heated to 95°C for 5 min, centrifuged for 2 min at 15 000× g, and hybridized under a 22 × 22 mm glass coverslip for 16 h in a 50°C water bath. Following hybridization, slides were washed and dried as described above.

Scanning and Analysis

Slides were scanned at resolution of 10 µm using a ScanArray® 4000 (Packard Bioscience, Meriden, CT, USA). A power setting of 80% was used for both the 635- and 532-nm lasers. The photomultiplier tube was set at 60% and 70% for Cy5 and Cy3, respectively. Images

were saved as 16-bit TIFF files and imported into Genepix™ Pro analysis software (Axon Instruments, Union City, CA, USA). Data were further processed by subtracting the mean background for each element on the microarray. All figures represent a red-green, square root transform false coloring scheme.

RESULTS AND DISCUSSION

We designed a series of experiments to assess the utilization of a Cy dye spike to identify common printing defects normally revealed after hybridization. Here we refer to DNA attached

to the substrate as the target and fluorescently labeled DNA in solution as the probe. Initial feasibility testing was conducted using 20 pmol/µL of a 30-bp oligonucleotide target spiked with dCTP-coupled Cy dye at various concentrations. The Cy3 dye spike was detectable at concentrations of 10 pmol/µL (Figure 1A), 1 pmol/µL (Figure 1B), or 0.1 pmol/µL (Figure 1C). Oligonucleotide alone showed no fluorescence (Figure 1D). The Cy5 spike generally showed a reduced intensity when compared to the Cy3 spike at identical concentrations (data not shown). Concentrations of 0.1 pmol/µL dCTP-Cy3 were easily detect-

Figure 3. Identification of spot morphology defects. (A and C) Oligonucleotide target arrayed with 0.02 pmol/µL dCTP-Cy3 and scanned before washing. (B and D) Panels A and C following washing and hybridization with Cy3- and Cy5-labeled II-23 T-cell hybridoma cDNA probe. (E) A pixel intensity plot across the white horizontal line in panel C (black trace) and panel D (gray trace). The Cy3 spike successfully identified ringing before hybridization (C, D, and E). Defects from a dust filament on the tip of arraying pin (A and B) are identified using the dCTP-Cy3 spike.

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ed under our scanning conditions. Slides were scanned, processed according to the prehybridization protocol, and scanned once again (Figure 1E-H). Following washing, no detectable fluorophore remained at concentrations of 1 and 0.1 pmol/ μ L. In addition, dye spikes were detectable at less than 1% of the original intensity for the 10 pmol/ μ L dilution (Figure 1E), indicating that dCTP-Cy3 shows virtually no covalent interaction with the substrate and is easily solubilized by the aqueous prehybridization buffer. We chose to use Cy3 as our spike for the remaining experiments because of its increased signal intensity at all concentrations and its known stability versus Cy5.

Next, the dCTP-Cy3 spike was tested for interference with the attachment of oligonucleotide targets. Oligonucleotide antisense and sense targets were arrayed either in the presence of 0.5 pmol/ μ L dCTP-Cy3 spike (Figure 2A, top and middle panel, respectively) or in the absence of fluorescent spike (Figure 2A, bottom panel). Slides were then scanned (Figure 2A), processed according to the prehybridization protocol, and scanned again (Figure 2B). Finally, slides were hybridized using equal molar amounts of Cy3 and Cy5 end-labeled antisense probe (Figure 2C). Visual comparison of hybridized spots containing the quality-control spike to those spots in which it was absent reveals that the addition of Cy3 spike had no effect on either spot size or spot morphology (compare Figure 2C, middle panel, to Figure 2C, bottom panel). In general, a linear decrease in the hybridized signal intensity is seen when decreasing amounts of oligonucleotide targets are arrayed (3). The median values of the Cy3 and Cy5 hybridization signal intensities from adjacent rows of 15 spotted elements were calculated for either target containing spike (Figure 2C, middle panel, row 5) or target not containing spike (Figure 2C, bottom panel, row 1). The Cy3 hybridization intensity was 1344 ± 360 relative fluorescence units (RFU) for target not containing the spike and 1173 ± 341 RFU for target containing the spike. Similarly, the Cy5 hybridization intensity was 829 ± 258 RFU for target not containing the spike and 829 ± 341 RFU for target containing the

spike. The Cy3 hybridization intensity data suggest that the dCTP-Cy3 spike may have reduced the oligonucleotide attachment and/or hybridization efficiency by 13%. However, this difference is within the standard deviation of hybridization intensity for these spotted elements and may be a result of "sub-array" variation (a trend of replicate gene elements showing hybridization intensity variations based solely on their array location). Therefore, we would consider the Cy3 hybridization intensity values to be essentially identical and conclude that the dCTP-Cy3 spike does not interfere with oligonucleotide attachment or hybridization. Ongoing hybridization experiments within our facility have further validated this conclusion (data not shown).

Using an array similar to the one described in the preceding paragraph, we studied the ability of a Cy3 spike to mimic the spot size seen before and after hybridization (data not shown). The spot size, as determined by our analysis software, was compared for 75 elements varying in size from 70 to 130 μ m. The correlation of spiked spot diameter to hybridized spot diameter was 0.96. Figure 3 shows a parallel experiment in which cDNA probe generated from II-23 T-cells was hybridized to an oligonucleotide array that contained 0.02 pmol/ μ L dCTP-Cy3 spiked into each target. Careful visual inspection of pre- and posthybridization (Figure 3, A and B) images shows that our quality-control spike successfully identified spot morphology defects such as catastrophic streaking caused by debris collecting on the quill pin. In addition, the spike was successful in predicting the extent of spot "ringing" following hybridization (Figure 3, C and D). This visual observation was confirmed by generating a pixel intensity plot in which pre-hybridized spots showed profiles similar to post-hybridization spots (Figure 3E).

We have found that the dCTP-Cy3 molecule is particularly difficult to remove from spotting pins at high concentrations. Therefore, we tested our working concentration of 0.02 pmol/ μ L dCTP-Cy3 to assure that no carryover effect was observed. First, a mixture of spotting buffer and oligonucleotide was arrayed. Pins were then washed with

three cycles of 10 s in water, 10 s in 70% ethanol, 10 s in water, and 15 s in vacuum. The same pins were used to array a mixture of spotting buffer, oligonucleotide, and dCTP-Cy3, followed by washing as described above. Finally, a fresh mixture of spotting buffer and oligonucleotide was arrayed. Comparing mean signal intensities of these arrayed spot series, we found no evidence that 0.02 pmol/ μ L dCTP-Cy3 causes cross contamination (data not shown).

Using dCTP-Cy3 is nondestructive, fast, and inexpensive. The addition of dCTP-Cy3 to a cDNA or oligonucleotide target library can be easily included as part of the concentration normalization and spotting buffer resuspension process. For a library of 10 000 ready-to-spot DNA elements in 10 μ L spotting buffer, the one-time addition of dCTP-Cy3 spike could potentially result in the fabrication of 1000 slides that contain individual quality-control measurements for each spot. Following a production run, slides could be scanned, batch-analyzed, and then discarded or stored for later use based on the facility standards. In contrast to staining methods, defects on every slide can be identified without reducing array performance and without assuming the quality of an entire print run based on only one or two assayed slides. At a final concentration of 0.02 pmol/ μ L, the library described above would require a total of only 2 nmol dCTP-Cy3, resulting in a total cost of approximately \$0.03 US/slide.

The combinations of different spotting environments, spotting buffers, arrayed products, slide chemistries, and deposition robots ensure that no two array facilities will fabricate microarrays in the exactly the same manner. The utility of our technique may ultimately be curtailed by this diversity. For example, the ability to remove dCTP-Cy3 during prehybridization washes is yet to be proven with cDNA array systems that typically require ultraviolet cross-linking or baking to attach DNA to substrate. Similarly, the ability to track fine morphology defects such as ringing may not be feasible in salt-deficient spotting buffers such as DMSO. Any limitations of the dCTP-Cy3 spike could potentially be avoided by substitution of an alternative fluorescent mol-

ecule. A dCTP-Cy3 spike will most likely benefit the many technology platforms based on chemistries that utilize a covalent bond to attach DNA to glass-modified substrates (2,4,5,7,9,11–14,16,17,19,20).

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