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Assessment of Fluorescence Resonance Energy Transfer for Two-Color DNA Microarray Platforms

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Two-color DNA microarray platforms are widely used for determining differential amounts of target sequences in parallel between sample pairs. However, the fluorescence (or Förster) resonance energy transfer (FRET) between two fluorophores can potentially result in the distortions of the measured fluorescence signals. Here we assessed the influence of FRET on the two-color DNA microarray platform and developed a reliable and convenient method for the correction of FRET distortion. Compared to current methods of normalization based on the statistical analysis and the hypothesis that only a small part of target sequences are differentially presented between sample pairs, our FRET correction method can recover the undistorted signals by the compensation of fluorescence emission, without considering the number of target sequences differentially presented. The correction method was validated with samples at different target ratios and with microarrays spotted in different probe concentrations. We also applied the FRET correction method to gene expression profiling arrays, and the results show that FRET was present when the content of target sequence was beyond a threshold amount and that the process incorporating our FRET correction method can improve the reliability of the gene expression profiling microarray platform in comparison with the current process without FRET correction.

DNA microarrays have been used widely for high-throughput explorations of genome structure, gene expression change, and gene functioning in basic molecular biological research,[1−3] clinical diagnosis,[4,5] and drug discovery.[6−8] One-color and two-color procedures are available for microarray analysis. In the one-color procedure, a single sample labeled with a single fluorophore is hybridized to each microarray having a well-defined pattern of DNA probes affixed to its surface, whereas in the two-color procedure, each of two samples (e.g., treatment and control) is labeled with one distinct fluorophore (e.g., Cy5 and Cy3) and a mixture of the labeled target samples is reacted with the same microarray[9] (Figure 1A). The emissions of the different fluorophores are acquired by two channels of a fluorescence scanner, and the ratio of the two fluorescent labels present on each probe spot is determined. The two-color procedure allows direct comparison of the sample pairs hybridized onto the arrayed probes and minimizes interexperimental variability.[10]

As both fluorophores are present, under some conditions, their individual fluorescence emissions cannot be detected independently because of the potential distortion due to FRET. When spectral overlap exists between the emission spectrum of the donor fluorophore (shorter excitation wavelength fluorophore) and the excitation spectrum of the acceptor fluorophore (longer excitation wavelength fluorophore) and the two fluorophores are physically close, typically within the 1−10 nm range, some of the energy held by the donor can be dissipated by nonradiative FRET to the acceptor, as well as by the normal radiative emission of

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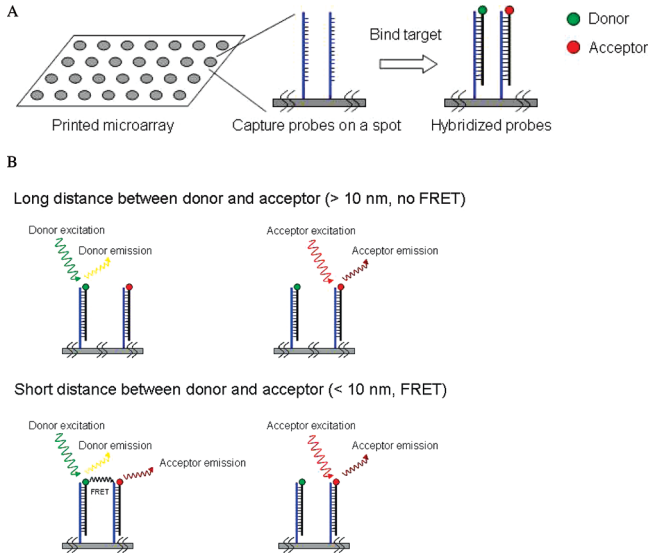

Figure 1. (A) Construction of two-color DNA microarray. The probes on a microarray are used to capture the targets in the mixture of acceptor-labeled and donor-labeled samples. (B) Fluorescence detection of two-color DNA microarray. The energy of the excited donor can be emitted by the radiative pathway and transferred nonradiatively to the acceptor when FRET occurs. FRET will not occur when the fluorophores are excited at the acceptor excitation wavelength.

fluorescence photons[11−17] (Figure 1B). Typically the donor channel (excitation, donor excitation wavelength; emission, donor emission wavelength) of the fluorescence scanner can only detect the donor emission and not the acceptor emission, and FRET will result in a decrease in the detected emission of the donor fluorophore.[18,19] The acceptor emission from the acceptor channel (excitation, acceptor excitation wavelength; emission, acceptor emission wavelength) will not be distorted by FRET because it does not occur when the fluorophores are excited at the acceptor excitation wavelength[20] (Figure 1B). Hence, the measured ratio of the acceptor to the donor will increase under conditions during which FRET occurs.

Some statistical methods, which are based on the adjustment of the distribution of the intensity log ratios,[21−23] have a potential effect on the FRET correction. However, they are mathematical manipulations of the distorted data and thus cannot improve the quality of analyzed microarray data. In addition, they force the distribution of the intensity log ratio to zero for each microarray and thus fail to correct for the FRET distortion on microarrays where the amounts of most target sequences are different between sample pairs. Physical methods have been developed to correct for FRET distortion in studies on living cells by the recovery of undistorted signals. However, these methods also suffer from various shortcomings, requiring previous measurement of fluorescence lifetime[24] or fluorescence spectral analysis of the samples[25] and thus are not suitable for microarray analysis. On a solid substrate, FRET distortion has been corrected for the determination of hybridization efficiency; however, the intermolecular FRET, which may be present on two-color DNA microarrays (e.g., gene expression profiling microarrays), is not assessed independently, and the process is complex because some standard samples should be prepared just to determine system-dependent factors.[26]

In this paper, we assessed the influence of intermolecular FRET on two-color DNA microarray platforms and developed a reliable and convenient method which utilizes the emission from the FRET channel (excitation, donor excitation wavelength; emission, acceptor emission wavelength) to compensate the donor emission and to eliminate the distortion due to FRET on two-color microarray platforms. Using customized calibration DNA microarrays, we demonstrated that FRET does occur during two-color DNA microarray experiments, and that with FRET correction, the ratio of fluorophore-labeled target sequences can be measured accurately. In comparison with the process for microarray-based gene expression profiling analysis without FRET correction, our procedure incorporating FRET correction can effectively account for FRET distortions, decrease the number of false positive genes detected in the analysis, and thus improve the reliability of this important analytical platform.

MATERIALS AND METHODS

Oligonucleotide Synthesis. For this study, a Cy3 (excitation max at 550 nm, emission max at 570 nm, donor) fluorophore and a Cy5 (excitation max at 649 nm, emission max at 670 nm, acceptor) fluorophore were used. Three oligonucleotides (oligo-1) all with the sequence 5′-TCCGTCATCGCTCAAG-3′ were synthesized as target sequences, one with Cy5 attached to the 3′ terminal ((oligo-1)-Cy5), one with Cy3 attached to the 3′ terminal ((oligo-1)-Cy3), and one with no fluorophore attached ((oligo-1)-nodye). The oligonucleotide ((oligo-2)-NH_2) with sequence 5′-CTTGAGCGATGACGGATTTTTTTTTTTTTTTT-3′ was coupled to an aldehyde-activated glass surface as the probe by the 3′ terminal amidocyanogen. The 5′ half of this oligonucleotide, which is complementary to oligo-1, is held by a poly-T tail away from the glass surface for free hybridization. All oligonucleotides were synthesized using basic phosphoramidite chemistry and purified by high-performance liquid chromatography (TaKaRa Biotechnology, Dalian, China).

Calibration DNA Microarray Construction. The oligonucleotide (oligo-2)-NH_2 was diluted in DNA spotting buffer (CapitalBio, Beijing, China) to fabricate the (oligo-2)-NH_2 array with DNA concentrations ranging from 0.05 to 6.40 μM in eight

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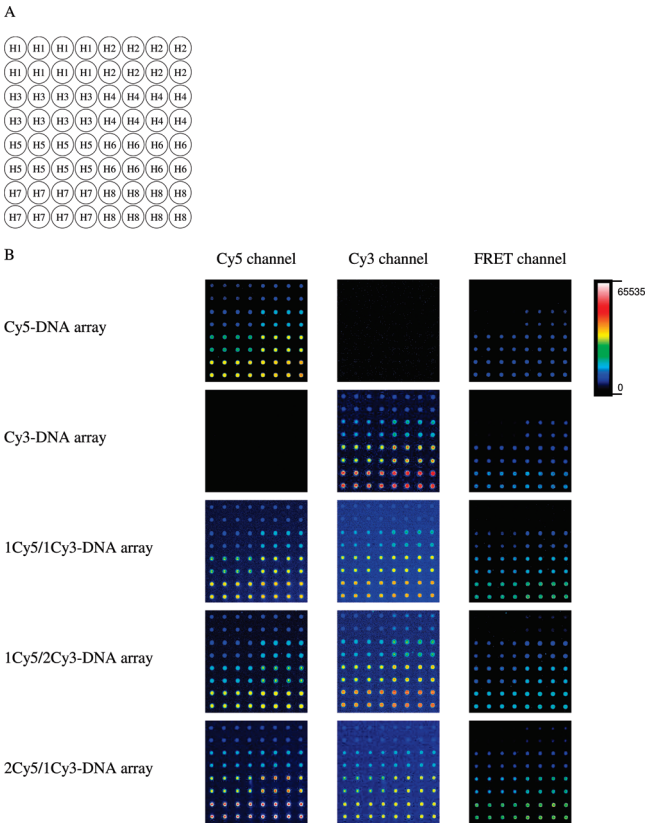

Figure 2. Spot pattern and images of the calibration DNA microarrays. (A) Pattern of the oligonucleotide (oligo-2)-NH_2 printed in eight subarrays. H1, 0.05 μM; H2, 0.10 μM; H3, 0.20 μM; H4, 0.40 μM; H5, 0.80 μM; H6, 1.60 μM; H7, 3.20 μM; H8, 6.40 μM. (B) Scanned microarray images. The respective images were obtained by scanning Cy5-DNA array, Cy3-DNA array, 1Cy5/1Cy3-DNA array, 1Cy5/2Cy3-DNA array, and 2Cy5/1Cy3-DNA array through the Cy5 channel, the Cy3 channel, and the FRET channel. The excitation power was set to 80, and the PMT gain was set to 630 for the Cy5 channel, the excitation power 80 and the PMT gain 630 for the Cy3 channel, and the excitation power 80 and the PMT gain 630 for the FRET channel.

subarrays (Figure 2A), and with four such arrays printed on one glass slide. The microarrays were prepared on aldehyde-activated glass slides using a SmartArrayer-48 microarray spotter (both CapitalBio).

Five mixed oligonucleotide solutions with a final oligo-1 concentration of 3.00 μM, comprising the concentration ratios of (oligo-1)-Cy5 to (oligo-1)-Cy3 to (oligo-1)-nodye of 1:0:2, 0:1:2, 1:1:1, 2:1:0, and 0.5:1:1.5, were prepared in microarray hybridization buffer I (5× Denhardt's solution, 0.2% SDS, 3× SSC in ultrapure water). A volume of 12 μL of each mixture was then hybridized to the (oligo-2)-NH_2 arrays to construct Cy5-DNA array, Cy3-DNA array, 1Cy5/1Cy3-DNA array, 2Cy5/1Cy3-DNA array, and 1Cy5/2Cy3-DNA array, respectively.

After hybridization at 42 °C for 2 h, the microarrays were washed with two consecutive wash solutions (0.2% SDS, 2× SSC at 42 °C for 4 min, and 0.2× SSC at 42 °C for 4 min). The dried microarrays were then scanned using a modified LuxScan-10K/A dual-channel laser confocal microarray scanner (CapitalBio), which was equipped with a third channel (a new combination of the laser in Cy3 channel and the emission filter in Cy5 channel) for the detection of the FRET emission signal. The fluorescence signals were collected from three channels of the microarray scanner, including the Cy3 (donor) channel (excitation = 532 nm, emission = 570 nm), the Cy5 (acceptor) channel (excitation = 640 nm, emission = 675 nm), and the FRET channel (excitation = 532 nm, emission = 675 nm). The microarray images were generated and analyzed by LuxScan v3.0 imaging and analysis software (CapitalBio).

Microarray-Based Gene Expression Profiling Analysis. Gene expression profiling analysis was performed using a 22k human genome 70-mer oligonucleotide microarray (CapitalBio).[10] The probes were printed on amino-silanized glass slides, each at an identical concentration of 20 μM. Universal human reference RNA (Stratagene, San Diego, CA) was used as both the treatment and control samples. The labeled DNA (Cy5-labeled treatment and Cy3-labeled control samples) was synthesized with 1 μg of total RNA, and two labeled samples were mixed into 80 μL hybridization solution II (5× Denhardt's solution, 0.2% SDS, 3× SSC, 25% formamide in ultrapure water). After the hybridization at 42 °C overnight and washing with two consecutive wash solutions (0.2% SDS, 2× SSC at 42 °C for 4 min, and 0.2× SSC at 42 °C for 4 min), the dried microarrays were scanned using the three channels of the modified LuxScan-10K/A microarray scanner, and the microarray images were generated and analyzed by LuxScan v3.0 imaging and analysis software. A normalization based on a "LOWESS" (locally weighted scatter plot smooth) program was applied to the microarray data before and after FRET correction. Genes with intensity (Cy3 or Cy5) >1500 were regarded as the expressed ones. To ensure the technical reproducibility, three experiments were performed, each of which involved a set of dye swap tests. We designed the self-to-self experiment in which the same biological sample was divided equally into two (the treatment and control samples), and the mixture of the Cy5-labeled treatment and Cy3-labeled control samples was hybridized to the same microarray. This experimental design was used to validate the reliability of the two-color microarray platform, as it minimizes the biological variability. In dye swap tests, the treatment sample was labeled with Cy3 and the control sample was labeled with Cy5. As the treatment and control samples were obtained from the same biological origin, the dye swap test was a technical reproduction of the former experiment.

FRET Correction Method for DNA Microarray. When FRET occurs between donor and acceptor fluorophores on a microarray, the donor signal from the donor channel will be distorted due to the nonradiative FRET from excited donor to acceptor. The loss of the donor emission from the donor channel can be determined by I_{FRET}/G, where G is defined as the ratio of the sensitized acceptor emission from the FRET channel (I_{FRET}) to the loss of affected donor emission from the donor channel due to FRET. Thus, the entire donor emission $I_{EntireDonor}$, which is corrected for the FRET effect, can be determined by the following equation:

$$I_{EntireDonor} = I_{Donor} + \frac{I_{FRET}}{G} \quad (1)$$

where I_{Donor} is the actual donor emission from the donor channel. The acceptor emission from the acceptor channel will not be distorted by the FRET effect in the presence of the donor. So the entire acceptor emission $I_{EntireAcceptor}$, can be directly determined by the following equation:

$$I_{\text{EntireAcceptor}} = I_{\text{Acceptor}} \quad (2)$$

where I_{Acceptor} is the actual acceptor emission from the acceptor channel.

Because of the crosstalk among the detection channels, the actual acceptor emission I_{Acceptor}, the actual donor emission I_{Donor}, and the sensitized acceptor emission I_{FRET} cannot be measured directly under some conditions. In a typical two-color microarray experiment, there is no emission from the acceptor channel when detecting a spot containing only the donor and no emission from the donor channel when detecting a spot containing only the acceptor. Crosstalk correction could be performed by the following equations:[20]

$$I_{\text{FRET}} = I_{\text{DA}} - dI_{\text{DD}} - aI_{\text{AA}} \quad (3)$$

$$I_{\text{Donor}} = I_{\text{DD}} \quad (4)$$

$$I_{\text{Acceptor}} = I_{\text{AA}} \quad (5)$$

where d (donor crosstalk factor) equals the ratio of signal from the FRET channel to that from the donor channel in a spot with only donor present, and a (acceptor crosstalk factor) equals the ratio of signal from the FRET channel to that from the acceptor channel in a spot with only acceptor present, both of which are approximately constant in a given two-color microarray system (fluorophores, fluorescence scanner, and scanning parameters are unaltered). I_{DD}, I_{AA}, and I_{DA} are the detected emission from the donor channel, the acceptor channel, and from the FRET channel, respectively.

Combining eqs 1−5, $I_{\text{EntireDonor}}$ and $I_{\text{EntireAcceptor}}$ can be determined by the following equations:[26]

$$I_{\text{EntireDonor}} = I_{\text{DD}} + \frac{I_{\text{DA}} - dI_{\text{DD}} - aI_{\text{AA}}}{G} \quad (6)$$

$$I_{\text{EntireAcceptor}} = I_{\text{AA}} \quad (7)$$

The correction factor G can be measured and calculated by various methods, such as the determination of the quantum yield,[20,27] the performance of photobleaching,[28,29] or the preparation of two or more different conjugates that contain both a donor and an acceptor.[26,30] However, these methods are not suitable for two-color microarray analysis. Here we developed a novel method to calculate G if we define the entire signal ratio R as the proportion of the acceptor signal (in the absence of the donor) to the donor signal (in the absence of the acceptor). Combining eq 6 and eq 7 allows us to yield eq 8:

$$R = \frac{I_{\text{AA}}}{I_{\text{DD}} + (I_{\text{DA}} - dI_{\text{DD}} - aI_{\text{AA}})/G} \quad (8)$$

The system-dependent factors d, a, and G relate to the fluorophores, the fluorescence scanner, and the scanning parameters, respectively, and remain constant in a given experimental system.[20,28] In particular, the value of R should not change with different degree of FRET effect if the amount ratio of two fluorophores is unaltered in a given two-color microarray system. In this paper, we calculated the factors d and a from the experimental measurements and calculated the values of factors G and R using a nonlinear programming method based on eq 8.

In a system where the same fluorophores and fluorescence scanner are used (kept constant) and only the scanning parameters are varied, the ratio (K) of the factor G to the factor d will remain constant, and conveniently, the correction factor G can be determined by eq 9:

$$G = Kd \quad (9)$$

where K relates to the fluorophores and the fluorescence scanner and is independent of the scanning parameters of the scanner. Equation 9 provides a novel and convenient method for the determination of the factor G, which is particularly suitable for the microarray analysis. The detailed derivation of eq 9 is presented in Supporting Information.

Importantly, eq 6 and eq 7 can be used for the accurate measurements of entire fluorescence signals eliminating the distortion due to FRET in the two-color microarray experiment, where no emission from the acceptor channel for the donor-only spot and no emission from the donor channel for the acceptor-only spot can be detected, regardless of whether FRET occurs or not.

RESULTS

Determination of the System-Dependent Factors for Calibration DNA Microarrays. For the FRET correction in common two-color microarray platforms, three system-dependent factors should be determined, including the donor crosstalk factor d, the acceptor crosstalk factor a, and the correction factor G. As no signals were detectable from the Cy3 channel for Cy5-DNA array or from the Cy5 channel for Cy3-DNA array, as seen in Figure 2B, the FRET correction can be performed following the methods described in Materials and Methods. According to the definitions provided, a can be calculated by the ratio of the signal from the FRET channel to the signal from the Cy5 channel on Cy5-DNA array in Figure 2B and equals 0.056 ± 0.007 for this system, while d can be calculated by the ratio of the signal from the FRET channel to the signal from the Cy3 channel on Cy3-DNA array in Figure 2B and equals 0.058 ± 0.006 for this system. This system involves the Cy3−Cy5 fluorophore pair, the LuxScan-10K/A microarray scanner, and the scanning parameters (excitation power 80 and PMT gain 630 for the Cy5 channel, excitation power 80 and PMT gain 630 for the Cy3 channel, and excitation power 80 and PMT gain 630 for the FRET channel).

Having determined the crosstalk factors d and a and measured the signals from three channels for all the spots on 1Cy5/1Cy3-DNA array (Figure 2B), we can calculate the correction factor G by the nonlinear programming method in which we propose to minimize the relative standard deviation of the entire signal ratio R calculated by eq 8 because R should remain stable as the amount ratio of two fluorophores is unaltered. The correction

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factor G equals 0.33, and the factor R equals 0.63. Here the value of R for 1Cy5/1Cy3-DNA array equals the system transfer factor, which is defined as the ratio of the acceptor signal (in the absence of the donor) to the donor signal (in the absence of the acceptor) for equal amounts of the donor and acceptor fluorophores in a given two-color microarray system (with defined fluorophores, fluorescence scanner, and scanning parameters). The system transfer factor can also be calculated by the linear regression analysis between the Cy5 signals on the Cy5-DNA array and the Cy3 signals on the Cy3-DNA array (Figure S1 in Supporting Information). The slope of the regression line is 0.65, which is close to the value determined by the nonlinear programming method, and the linear correlation coefficient is 0.99 (close to 1.00), demonstrating that the factor R determined by the nonlinear programming method is accurate and reliable.

In order to validate that the ratio (K) of G to d is independent of the scanning parameters, the microarray images of the 1Cy5/1Cy3-DNA array obtained by the microarray scanner with five sets of scanning parameters were analyzed by the same nonlinear programming method. The results are shown in Table S1 of Supporting Information. The line regression analysis shows that the correlation coefficient is 0.92 (close to 1.00) between d and G when the intercept is set to zero. Thus the factor K remains approximately constant on microarray platforms when the same fluorophores (constant fluorescent properties) and the same microarray scanner are used. The factor G can be calculated conveniently by the measured value d and the constant K when only the scanning parameters are changed.

FRET Correction on Customized DNA Microarrays. Next, we compared the signals on the Cy3-DNA array with the signals on three two-color microarrays, including 1Cy5/1Cy3-DNA array, 1Cy5/2Cy3-DNA array, and 2Cy5/1Cy3-DNA array. The microarray images are shown in Figure 2B. On these microarrays, the Cy3 amounts were constant and the Cy5 amounts were different on corresponding spots because the same proportion of (oligo-1)-Cy3 and different proportion of (oligo-1)-Cy5 were present in the hybridization solutions and identical hybridization conditions were used throughout.

The linear regression analysis of the measured Cy3 signals is shown in Figure 3A (black lines). The Cy3 intensities on each of three two-color microarrays are approximately equal to those on the Cy3-DNA array when the probe concentrations are low (i.e., Cy3 intensities are low) because the low densities of fluorophores on the spots preclude their FRET interaction. As the probe concentrations increase and Cy3 intensities become higher, the Cy3 intensities on each of three two-color microarrays are much lower than those on the Cy3-DNA array. When the probe concentration increases, the distance between Cy3 and Cy5 decreases. As a consequence, the shortened distance may result in higher FRET efficiency and greater loss of donor emission. From Figure 3B, the slopes of three regression lines are all below 1.00.

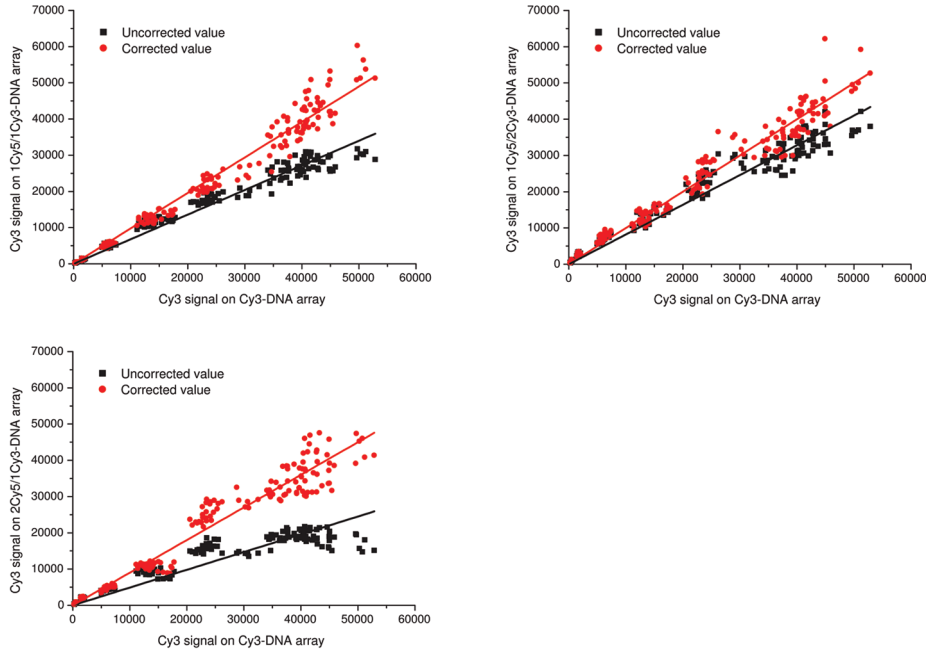
Comparing the slopes of the regression lines from Figure 3B, there is a decrease of the slope as the proportion of Cy5 fluorophores increases. When the Cy3 amounts are constant and Cy5 amounts increase, more Cy3 fluorophores are in complex with the Cy5 fluorophores and participate in FRET, and the distances between Cy3 and Cy5 become shorter. This results in greater loss of the Cy3 emission.

The linear regression analysis between the corrected Cy3 signals on each of three two-color microarrays and the measured Cy3 signals on the Cy3-DNA array is shown in Figure 3A (red lines). The slopes of the regression lines are all close to 1.00 (Figure 3B), which indicates that the FRET correction can compensate the loss of the Cy3 emission due to FRET on two-color microarrays regardless of whether FRET occurs and the amount ratio of Cy5 to Cy3.

The amount ratio of Cy5 to Cy3 on each spot was also measured. The amount ratio equals the value of I_{Cy5}/I_{Cy3} divided by the system transfer factor, and A is calculated by $\log_2(I_{Cy5} \cdot I_{Cy3})$. Here I_{Cy5} is I_{AA} and I_{Cy3} is I_{DD} for the calculation of an uncorrected value, and I_{Cy5} is $I_{EntireAcceptor}$ and I_{Cy3} is $I_{EntireDonor}$ for the calculation of a corrected value. On 1Cy5/1Cy3-DNA, 1Cy5/2Cy3-DNA, and 2Cy5/1Cy3-DNA arrays, amount ratios of Cy5 to Cy3 fluorophores are 1:1, 1:2, and 2:1, respectively. Theoretically, the calculated ratios from the same microarray should be constant. From Figure 3C, the uncorrected ratios of Cy5 to Cy3 remain approximately constant when the values of A are less than 25; however, when the values of A are larger than 25, the ratios of Cy5 to Cy3 increase as the values of A increase on each microarray. When the distance between Cy5 and Cy3 is less than 10 nm, the FRET efficiency increases with increasing probe density and thus the loss of the Cy3 emission from the Cy3 channel will also increase, whereas the Cy5 emission will not be affected. Yet after correction for the FRET effect, the ratios from each microarray remain approximately constant, illustrating that the correction method can effectively eliminate FRET distortion of signal values. The corrected values smoothed by the LOWESS fitting method shown in Figure 3C are also close to the true amount ratios of target sequences on 1Cy5/1Cy3-DNA, 1Cy5/2Cy3-DNA, and 2Cy5/1Cy3-DNA arrays (i.e., 1.0, 0.5, and 2.0, respectively). These various results demonstrate that the correction method can provide accurate measurements of the differential amounts of target sequences between sample pairs. In contrast, the statistical methods developed by others, which force the distribution of the intensity log ratios to zero for each microarray, are not accurate when the amounts of target sequences in sample pairs are significantly different (e.g., 1Cy5/2Cy3-DNA array and 2Cy5/1Cy3-DNA array).

Correction of Gene Expression Ratios For FRET Effects. Having demonstrated the FRET correction method using the specially constructed calibration microarrays, we applied it to normal gene expression profiling microarray to assess the FRET distortion and to validate our correction method for this platform. We designed self-to-self experiments which involved the alternative use of Cy3 and Cy5 labels to tag the treatment and control samples, ensuring that each sample was labeled with each dye and could be compared to its signal with the alternative dye. Ideally, the normalized ratios of Cy5 to Cy3 on all the spots, which present the differentially expressed fold, are evenly distributed around 1 and there is no expression difference to each expressed gene between the treatment and control samples; however, ratio values other than 1 can often be detected due to experimental and systematic variations. When the cutoff of the differentially expressed fold is closer to 1, the number of detected differentially

A



B

Microarray	Slope of regression line		Linear correlation coefficient	
	Uncorrected	Corrected	Uncorrected	Corrected
1Cy5/1Cy3-DNA array	0.68	0.98	0.97	0.98
1Cy5/2Cy3-DNA array	0.82	1.00	0.96	0.97
2Cy5/1Cy3-DNA array	0.49	0.90	0.89	0.96

C

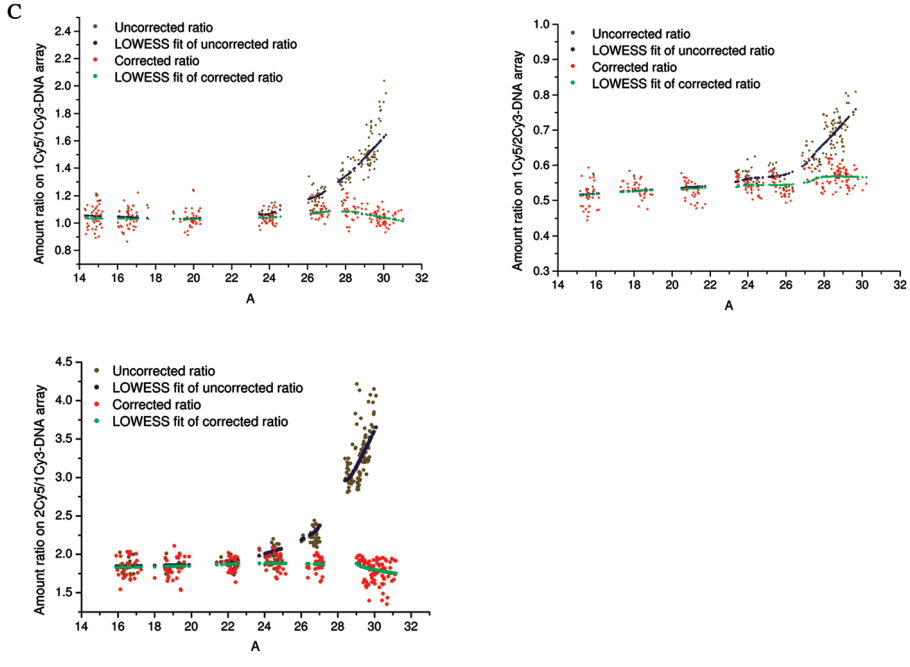


Figure 3. Fluorescence measurements of the constructed DNA microarrays and the correction of these measurements. All data were obtained using the same scanning parameters (the excitation power 80 and the PMT gain 630 for the Cy5 channel, the excitation power 80 and the PMT gain 630 for the Cy3 channel, and the excitation power 80 and the PMT gain 630 for the FRET channel). Thus, FRET corrections were performed using the same system-dependent factors. (A) Linear regression analysis of the Cy3 signals between each of the two-color microarrays and the Cy3-DNA array. The intercept of each regression line is made equal to zero. (B) Slope of the regression line and the linear correlation coefficient from the linear regression analysis of Cy3 signals. (C) Amount ratio versus *A* plot of the constructed DNA microarrays. The amount ratio is the value of I_{Cy5}/I_{Cy3} divided by the system transfer factor, and *A* is calculated by $\log_2(I_{Cy5} \cdot I_{Cy3})$.

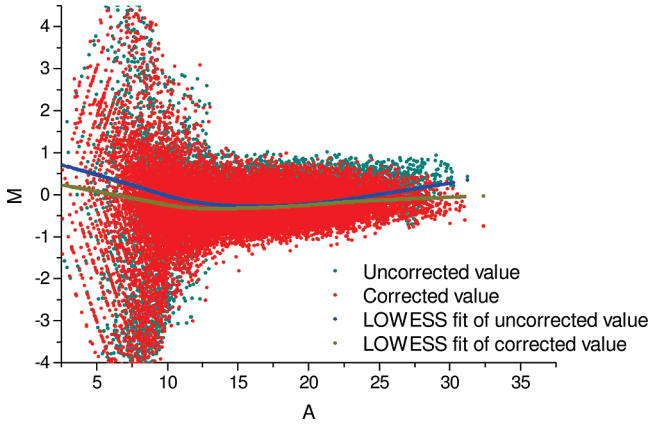


Figure 4. *M* versus *A* plot of a gene expression profiling microarray. The plot demonstrates both the distorted (uncorrected) *M* values (green dot) and the *M* values after the correction for the FRET effects (red dot). *M* is calculated by $\log_2(I_{Cy5}/I_{Cy3})$, and *A* is calculated by $\log_2(I_{Cy5} \cdot I_{Cy3})$.

expressed genes (false positive genes) will be greater. Hence, when the cutoff of the differentially expressed fold is the same, fewer differentially expressed genes indicate that the result has fewer false positive gene signals and thus the platform has higher reliability. Ideally, in a self-to-self experiment, the number of the differentially expressed genes is zero.

The "*M*−*A* plot" shown in Figure 4 is used to represent the expression profiling data, where *M* is calculated by $\log_2(I_{Cy5}/I_{Cy3})$ and *A* is calculated by $\log_2(I_{Cy5} \cdot I_{Cy3})$. The values of *M* increase as the intensity values of *A* increase when the values of *A* are larger than 25 because, after crosstalk correction, the signals of the corresponding spots obtained from the FRET channel become strong (data not shown). This indicates that FRET has occurred and the loss of Cy3 emission is present (a detailed explanation is presented in the section of calculation of FRET efficiency in Supporting Information). Here, for the FRET correction, we use the nonlinear programming method to determine the factor *G*. The values of *M* calculated by the corrected Cy3 signals become more stable than the values without FRET correction. These data illustrate that FRET distortion is present on microarray-based expression profiling platforms, especially at high intensity values when the densities of fluorophores are sufficiently high on a spot. Our method can eliminate this FRET distortion and thus obtain more accurate data for two-color analysis. Figure 4 also shows that the values of *M* increase as the intensity values of *A* decrease when the values of *A* are less than 15 because the background intensities of the images from the Cy3 channel are stronger than those from the Cy5 channel and the distortion of background will be more serious on the spots with lower intensities when local median and background subtraction are applied. However, as these genes would not be generally regarded as the expressed ones because of their low intensities, this effect will not distort the results.

In order to further examine our FRET correction on expression profiling analysis, three experiments incorporating a dye swap were performed, and the numbers of differentially expressed genes in each experiment were counted using both uncorrected and corrected methods (Table 1). Without considering the dye swap, there is no significant difference in the number of differentially expressed genes identified between the corrected group and the uncorrected group for the six microarrays (two microarrays in each of three experiments) when the differentially expressed cutoff is 2.0-fold ($P = 0.17$), which shows that the FRET effects do not cause serious errors in the determination of the number of differentially expressed genes at a higher cutoff value on this expression profiling platform. However, when a cutoff of 1.5-fold is used, the numbers of differentially expressed genes calculated by the corrected signals are less than those calculated by the uncorrected signals ($P = 0.01$). This result shows that the correction of FRET can decrease the number of the false positive genes detected on each microarray. Considering the dye swap, there is no significant difference in the number of differentially expressed gene between the corrected group and the uncorrected group when the cutoff is 1.5-fold ($P = 0.27$) or when it is set to 2.0-fold ($P > 0.05$). The distortion of FRET can be partly eliminated by incorporating dye swap experiments (i.e., technical reproduction in our experiments) and the data normalization using the LOWESS program. In comparison with the procedure without FRET correction, our procedure can provide more stable ratios and thus more accurate data for each microarray. After the data normalization of each microarray, the number of false positive genes with FRET correction is less than that without FRET correction when a cutoff of 1.5-fold is used. Thus, FRET correction can improve the reliability of the microarray-based gene expression profiling platform, particularly at lower cutoff values and in higher probe and target concentrations.

DISCUSSION

Theoretically, to eliminate FRET distortion in two-color microarray experiments, fluorophores with appropriate spectral properties should be chosen, or intermolecular distances beyond the range of FRET interaction should be employed. However, the fluorophores commonly used in two-color microarray experiments have a spectral overlap, and the distance between fluorophores cannot be rigidly controlled on the solid substrate of the microarray. Thus, the possibility of FRET distortion cannot be entirely and readily excluded. We have demonstrated that FRET distortion occurs frequently on two-color microarrays, particularly when the concentrations of targets or probes are high. Thus, it is necessary to correct for FRET distortion to ensure greater accuracy of the desired fluorescence signals. In Figure S2 of Supporting Information, we compared Cy3 signals between the Cy3-DNA array and the 1Cy5/1Cy3-DNA array and also compared Cy5 signals between the Cy5-DNA array and the 1Cy5/1Cy3-DNA array. From Figure S2A, it can be seen that, when the probe concentration is less than or equal to 0.8 μM, the Cy3 signals on the Cy3-DNA array are close to the Cy3 signals on 1Cy5/1Cy3-DNA array. When the probe concentration is greater than 0.8 μM, there is an obvious difference between the Cy3 signals on the Cy3-DNA array and the Cy3 signals on the 1Cy5/1Cy3-DNA array, which is caused by FRET. There is no obvious difference between the Cy5 signals on the Cy5-DNA array and the Cy5 signals on the 1Cy5/1Cy3-DNA array seen in Figure S2B, which indicates that the Cy5 signals are not affected by FRET. Moreover, from Figure S1, the linear correlation coefficient between the Cy5 signals on the Cy5-DNA array and the Cy3 signals on the Cy3-DNA array is 0.99, which indicates that the Cy5 signals on the Cy5-DNA array are linearly proportional to the Cy3 signals on the Cy3-DNA array.

Table 1. Numbers of Differentially Expressed Genes Detected in Three Gene Expression Profiling Experiments[a]

		experiment 1			experiment 2			experiment 3		
differentially expressed fold	method	array 1	dye swap array 2	intersection of array 1 and array 2	array 1	dye swap array 2	intersection of array 1 and array 2	array 1	dye swap array 2	intersection of array 1 and array 2
1.5	uncorrected	60	63	0	9	55	13	65	50	1
	corrected	46	38	0	8	29	10	48	41	0
2.0	uncorrected	0	3	0	1	2	1	0	0	0
	corrected	0	2	0	1	1	1	0	0	0

[a] Note: The software for microarray data analysis was provided by CapitalBio. Differentially expressed genes involve up-regulated genes and down-regulated genes. The corrected group is analyzed by the corrected Cy3 intensities, and the uncorrected group is analyzed by the uncorrected Cy3 intensities. When a gene is regarded as the expressed gene, both on array 1 and on dye swap array 2, and the average of differentially expressed values on both arrays is higher than the cutoff, this gene is counted in the "intersection of array 1 and array 2" column.

Unlike the statistical methods of others which cannot improve the quality of analyzed microarray data, our FRET correction method provides the recovery of undistorted (original) signals for subsequent data analysis. Hence the correction method can significantly improve the accuracy of two-color microarray platforms. Some statistical methods have a potential effect on the correction of the FRET distortion, but they are applicable only when the amounts of most target sequences in sample pairs are very close. Our FRET correction method is particularly applicable for the analysis of differential amounts of target sequences where most amount ratios of the acceptor to the donor are not 1:1. For the microarray-based gene expression profiling analysis, there are many experimental and systematic distortions because of this complexity. As our FRET correction method can eliminate the general distortion of FRET, we recommend its use, before statistical normalization of microarray data, for more accurate analysis of complex gene expression profiling. Table 1 shows that the FRET correction does not have a significant impact at a cutoff of 2.0-fold but does have an impact at a cutoff of 1.5-fold. Theoretically, the analysis with a lower cutoff value could detect genes with less expression difference between sample pairs and thus could increase the sensitivity of microarray platform. However, the distortion of differentially expressed values due to the FRET effect will result in more inaccurate determination and decrease the reliability under this condition. FRET correction can provide a more reliable and more sensitive analysis.

Our correction method is convenient because, after the system-dependent factors have been determined, only the signals from three channels need to be obtained and because the method can be used to process the entire array regardless of whether FRET occurs or not, and regardless of the amount ratios of Cy5 to Cy3 on different spots. The system-dependent factors are approximately constant when the same fluorophores, microarray scanner, and scanning parameters are used. When only the scanning parameters are varied, the factors d and a can be simply measured from the donor-only spot and the acceptor-only spot, respectively, and the factor G can also be conveniently calculated from eq 9. In some experimental systems, when the densities of fluorophores on a spot are high enough, the degree of fluorescence autoquenching will arise significantly. This may impact the quantum efficiencies; consequently, the crosstalk factors d and a may be variable. In our experiment when the concentrations of capture probes range from 0.05 to 6.40 μM, the coefficient of variation of crosstalk factor a is 12.50% and that of crosstalk factor d is 10.34%. So in this system, the autoquenching effect does not have a significant impact on the values of the crosstalk factors. However, when the autoquenching effect cannot be ignored in experimental systems or more accurate measurement is required, the crosstalk factors of each spot in a different intensity range can be determined independently according to matching fluorescence levels.[27] When the properties of fluorophores change due to the different fluorophore conditions, the factor G can also be calculated by the nonlinear programming method for more accurate FRET correction when only a small part of target genes are differentially expressed between sample pairs.

As the capture probes often exhibit inhomogeneous distributions on a spot of a microarray, the fluorophores will also exhibit inhomogeneous distributions. In practice, the analysis of a microarray is based on fluorescence intensities of a whole spot, thus FRET correction at each location is equivalent to the implementation on a whole spot when the factors d, a, and G are unaltered on a spot. If the variations of system factors cannot be ignored due to the inhomogeneous distributions of fluorophores on a spot, the FRET correction with appropriate factors at each location can provide more accurate analysis.

Because FRET is a distance-dependent interaction between appropriate fluorophores, the FRET distortion will be greater when the densities of fluorophores are higher in particular spots. Thus, to decrease the FRET effect on the detected fluorescence emission of the microarray, one method could be to rigidly control the concentrations of probes and targets; ideally by avoiding the overabundance of probes and targets, the attendant binding of excess levels of targets could be decreased. Under this condition, the sensitivity of current fluorescence scanners should be improved greatly and the process of constructing a DNA microarray should be optimized to increase the signal intensity and decrease the background noise. Yet, these solutions cannot be ideally implemented for current microarray platforms. In the gene expression profiling analysis using the commercial 22k human genome oligonucleotide microarrays, the exogenous spike-in mRNA samples from the yeast and the corresponding control probe spots printed on the microarray were employed. When 0.2 ng of spike-in mRNA was present in both 1 μg of treatment RNA sample and 1 μg of control RNA sample, the value of A (calculated by $\log_2(I_{Cy5} \cdot I_{Cy3})$) was about 25 and the FRET efficiency (calculated by eq S7 in Supporting Information) was close to zero. The results indicated that when the content of mRNA was below 0.2 ng there was no strong FRET interaction and the loss of donor emission can be ignored in our experimental system.

CONCLUSIONS

In this paper, we assessed the distortion of FRET in two-color microarray experiments and developed a reliable and convenient method for the FRET correction. Using both measurements of the amount ratios on constructed calibration microarrays and gene expression profiling analysis on standard microarrays, we demonstrated that intermolecular FRET interaction was present and could distort the fluorescence measurement on two-color DNA microarrays. We advanced the FRET correction method to two-color microarray platforms. The results show that the FRET correction method can provide accurate, quantitative and convenient measurements of the fluorescence intensities on the two-color microarray and provide a more reliable analysis for microarray-based gene expression profiling.

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SUPPORTING INFORMATION AVAILABLE

Additional experimental material. This material is available free of charge via the Internet at http://pubs.acs.org.

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