Cross-talk correction in dual-labeled fluorescent microarray scanning

Liqiang Wang (王立强), Zukang Lu (陆祖康), and Xuxiang Ni (倪旭翔)

State Key Lab of Modern Optical Instrumentation, Zhejiang University, Hangzhou 310027

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Cross-talk phenomenon in dual-labeled fluorescent microarray scanning is analyzed from cross-excitation and cross-emission. It is turned out that the spectral overlap of the fluorophores is crucial for cross-talk error, and this error can be corrected by an image subtraction method. The experiment was successfully applied to separate the Cy3 channel and the Cy5 channel in microarray scanning. The cross-talk error was reduced from more than 1% to about 0.1%.

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DNA microarray is a new and powerful tool that allows the simultaneous analysis of a large number of nucleic acid hybridization experiments in a rapid and efficient fashion. In a typical microarray experiment, the two mRNA samples to be compared are reverse transcribed into cDNA, labeled using two different fluorophores (usually a red fluorescent dye, Cy5, and a green fluorescent dye, Cy3), and then hybridized simultaneously to the glass slide[1,2].

To analyze the slide, a microarray reader uses a light source, a laser or lamp, to excite the fluorophores. A photomultiplier tube or CCD camera then detects the resulting fluorescence, and the system produces an image that shows the intensity of the two fluorophores[3]. By using two detectors in combination with a dichroic filter, it is possible to record the distribution of the two fluorescent labels simultaneously. However, because of the spectral overlap of the two fluorophores, each detector will generally detect light from more than one fluorophore. Hence, cross-talk phenomenon between the fluorescence channels occurs[4,5].

In order to reduce channel cross-talk error and improve the image quality, we have designed a microarray scanner to record the two fluorophores by scanning the microarray slide twice, using different laser wavelengths and detector filters[6]. The microarray scanner first scans the slide by the red laser (635 nm) to obtain the fluorescence image of Cy5 dyes, the green laser (532 nm) is blocked during this period; then the scanner scans the slide by the green laser to obtain the Cy3 image, the red laser now is blocked instead. Unfortunately, we find the channel cross-talk error is still not negligible. So it is necessary to find an image processing method to correct or reduce the cross-talk error[7,8].

A straightforward method for correcting the dual channel cross-talk error is based on image subtraction with a suitable coefficient. This coefficient represents the degree of cross-talk error. After image subtraction using this coefficient, the image intensity, which is a function of fluorophore concentration, should vary in the same manner as if the microarray slide was labeled with a single fluorophore. We now deduce this coefficient from cross-excitation and cross-emission between two different fluorophores.

A fluorophore is not only excited by the wavelength at its peak value, but also by the wavelength at certain range around the peak, which can extend into the area used by another fluorophore. From Fig. 1, it can be seen that Cy3 has an excitation peak (100% of relative intensity) at 550 nm, it is still excited near 2% at 635 nm which is the wavelength used to excite Cy5 in our scanner, Cy5 has an excitation peak at 649 nm, it is excited near 4% at 532 nm which is the wavelength used to excite Cy3.

Cross-emission is another factor which causes channel cross-talk. When emission spectra of two fluorophores overlap, emission from one channel will extend to another channel. Unfortunately, many commonly used fluorophore pairs have more or less overlapped emission spectra which pose the problem of cross-emission in most dual-labeled application. From Fig. 1, we can see that Cy3-Cy5 pair has considerable cross-emission.

Let S_1 and S_2 denote the fluorescence signals excited by wavelength λ_1 and λ_2, respectively. Assume that they are linear functions of the amount of fluorophores and the powers of the illuminating lights. S_1 and S_2 can be described by

$$S_1 = k \cdot \left\{ \rho_1 \varepsilon_1(\lambda_1) P_1 \int e_1(\lambda) T_1(\lambda) \mathrm{d}\lambda + \rho_2 \varepsilon_2(\lambda_1) P_1 \int e_2(\lambda) T_1(\lambda) \mathrm{d}\lambda \right\}, \quad (1)$$

$$S_2 = k \cdot \left\{ \rho_2 \varepsilon_2(\lambda_2) P_2 \int e_2(\lambda) T_2(\lambda) \mathrm{d}\lambda + \rho_1 \varepsilon_1(\lambda_2) P_2 \int e_1(\lambda) T_2(\lambda) \mathrm{d}\lambda \right\}, \quad (2)$$

where $\rho_{1,2}$ are the fluorophore concentrations, $\varepsilon_{1,2}(\lambda_{1,2})$ are the absorption coefficients of the fluorophores in wavelength λ_1 and λ_2, $P_{1,2}$ are the excitation powers of wavelength λ_1 and λ_2, $e_{1,2}(\lambda)$ are the emission spectra of the fluorophores, $T_{1,2}(\lambda)$ are the transmission spectra of the filters corresponding to wavelength λ_1 and λ_2. S_1 and S_2 also depend on the factors such as chemical environment of the fluorophores and fading due to long time exposure. We assume that all such factors are constant so that only fluorophore concentrations vary over the microarray slide, and k denotes this constant.

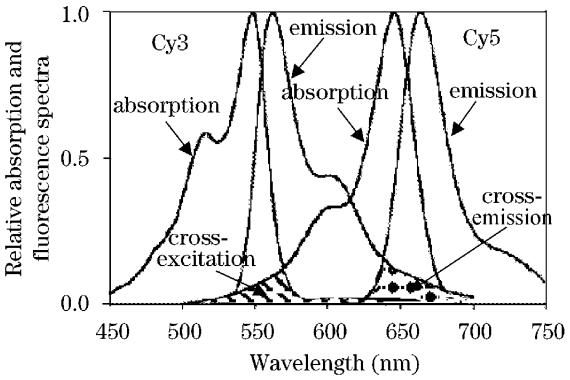

Fig. 1. Absorption and fluorescence spectra of Cy3 and Cy5 fluorophores.

From Eq. (1), we can see that S_1 depends on both ρ_1 and ρ_2. Although $\varepsilon_2(\lambda_1)$ is usually small, when ρ_2 is very high and ρ_1 is relatively low, S_1 will contain much part of fluorescence signal emitting from the unwanted fluorophore. S_2 suffers the same problem. So we need to find out a method which can eliminate this cross-talk error.

According to Carlsson and Mossberg[7], we rewrite Eq. (1) as

$$S_1 = F_1 + f_2, \tag{3}$$

where

$$F_1 = k \cdot \rho_1 \varepsilon_1(\lambda_1) P_1 \int e_1(\lambda) T_1(\lambda) \mathrm{d}\lambda, \tag{4}$$

and

$$f_2 = k \cdot \rho_2 \varepsilon_2(\lambda_1) P_1 \int e_2(\lambda) T_1(\lambda) \mathrm{d}\lambda. \tag{5}$$

Analogously, we can rewrite Eq. (2) as

$$S_2 = F_2 + f_1, \tag{6}$$

where f_1 and f_2 represent cross-talk values between the fluorophores. If, in a particular microarray slide, only one fluorophore is present, the fluorescence signal excited by wavelength λ_1 will be only F_1 and excited by λ_2 will be only f_1. In the later case, S_2 will be entirely unwanted cross-talk value. Define the cross-talk ratio α_1 as

$$\alpha_1 = \frac{f_1}{F_1} = \frac{\varepsilon_1(\lambda_2) P_2 \int e_1(\lambda) T_2(\lambda) \mathrm{d}\lambda}{\varepsilon_1(\lambda_1) P_1 \int e_1(\lambda) T_1(\lambda) \mathrm{d}\lambda}, \tag{7}$$

multiplying S_1 by α_1 and subtracting the result from S_2, we will correct the channel cross-talk because $S_2 - \alpha_1 S_1 = 0$. Analogously, we define the cross-talk ratio α_2 as

$$\alpha_2 = \frac{f_2}{F_2} = \frac{\varepsilon_2(\lambda_1) P_1 \int e_2(\lambda) T_1(\lambda) \mathrm{d}\lambda}{\varepsilon_2(\lambda_2) P_2 \int e_2(\lambda) T_2(\lambda) \mathrm{d}\lambda}. \tag{8}$$

If two fluorophores are co-localized, the fluorescence signals are given by Eqs. (1) and (2). After subtracting the cross-talk portion, the signals can be written as

$$\begin{aligned} S_1' &= S_1 - \alpha_2 \cdot S_2 \\ &= k \cdot \rho_1 P_1 \left[\varepsilon_1(\lambda_1) \int e_1(\lambda) T_1(\lambda) \mathrm{d}\lambda \right. \\ &\quad \left. - \frac{\varepsilon_1(\lambda_2)\varepsilon_2(\lambda_1) \int e_1(\lambda) T_2(\lambda) \mathrm{d}\lambda \int e_2(\lambda) T_1(\lambda) \mathrm{d}\lambda}{\varepsilon_2(\lambda_2) \int e_2(\lambda) T_2(\lambda) \mathrm{d}\lambda} \right], \end{aligned} \tag{9}$$

$$\begin{aligned} S_2' &= S_2 - \alpha_1 \cdot S_1 \\ &= k \cdot \rho_2 P_2 \left[\varepsilon_2(\lambda_2) \int e_2(\lambda) T_2(\lambda) \mathrm{d}\lambda \right. \\ &\quad \left. - \frac{\varepsilon_1(\lambda_2)\varepsilon_2(\lambda_1) \int e_1(\lambda) T_2(\lambda) \mathrm{d}\lambda \int e_2(\lambda) T_1(\lambda) \mathrm{d}\lambda}{\varepsilon_1(\lambda_1) \int e_1(\lambda) T_1(\lambda) \mathrm{d}\lambda} \right]. \end{aligned} \tag{10}$$

S_1' is independent on ρ_2 and S_2' is independent on ρ_1, they vary with fluorophore concentration in the same manner as if the microarray slide was labeled with a single fluorophore, except a constant factor. So we can correct cross-talk error by subtracting this portion. It depends on the absorption and emission spectra of the fluorophores and the transmission spectra of the filters.

Although Eqs. (7) and (8) are simple, it is very difficult to calculate α_1 and α_2 accurately because the absorption and emission spectra of fluorophores depend on many factors such as chemical solutions, temperature, PH value, and time etc.. Fortunately, we can obtain α_1 and α_2 through simple experiments.

We detected cross-talk error by the FMB Scanner Calibration Slide[9]. This slide contains two separate blocks of arrays in dilution series of Cy3 and Cy5 fluorescent dyes. Each block consists of 28 sets of two-fold dilutions of Cy3 or Cy5. Firstly, the slide was scanned by the green laser only. The acquired image was saved as image A (Fig. 2(a)). Then, the slide was scanned by the red laser only. The acquired image was saved as image B (Fig. 2(b)). Finally, the intensity of Cy3 and Cy5 signals from both scans was analyzed. Cross-talk ratio of Cy5 (α_{Cy5}) was calculated by measuring the average pixel intensity of the Cy5 (I_{Cy5}) in the highest non-saturating Cy3 column, subtracting the background B_{Cy5}, and dividing that by the corresponding Cy3 average pixel intensity I_{Cy3} subtracting the background B_{Cy3}. The reverse was done to measure Cy3 cross-talk ratio α_{Cy3}. So we rewrite Eqs. (7) and (8) as

$$\alpha_{\mathrm{Cy5}} = \frac{I_{\mathrm{Cy5}} - B_{\mathrm{Cy5}}}{I_{\mathrm{Cy3}} - B_{\mathrm{Cy3}}} \quad \text{(green laser scanned)}, \tag{11}$$

$$\alpha_{\mathrm{Cy3}} = \frac{I_{\mathrm{Cy3}} - B_{\mathrm{Cy3}}}{I_{\mathrm{Cy5}} - B_{\mathrm{Cy5}}} \quad \text{(red laser scanned)}. \tag{12}$$

According to the cross-talk ratio α_{Cy5} and α_{Cy3}, we corrected the cross-talk error using Eqs. (9) and (10). The results were listed in Table 1. The final images were shown in Figs. 2(c) and (d).

There were 8 columns of fluorophore dilutions, we found that the cross-talk ratios calculated by other columns were similar as that calculated by the highest non-saturating column. This verified again that the cross-talk ratio was independent on the fluorophore concentration, as Eqs. (7) and (8) show.

Table 1. Experiment Results of Cross-Talk Analysis

	Original Data		After Correction	
Laser	Green	Red	Green	Red
I_{Cy3}	53766	813	53754	136
I_{Cy5}	761	48119	34	48109
B_{Cy3}	74	88	73	87
B_{Cy5}	82	97	81	96
Cross-Talk (%)	1.26	1.51	−0.09	0.10

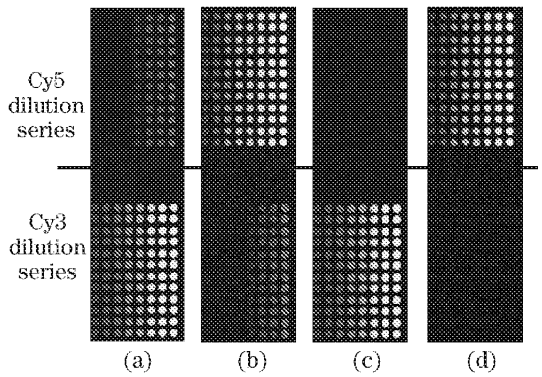

Fig. 2. The fluorescent image of Cy3 and Cy5 dilutions (only 8 columns shown). (a) Scanned by green laser only; (b) scanned by red laser only; (c) image (a) after cross-talk correction; (d) image (b) after cross-talk correction.

We defined the cross-talk ratio of our microarray scanner as that in Table 1. After scanning a new microarray slide labeled by Cy3 and Cy5, we used this ratio as subtraction coefficient in Eqs. (9) and (10). The cross-talk error was reduced from more than 1% to about 0.1%.

In conclusion, we described an image subtraction method to reduce channel cross-talk in dual-labeled fluorescent microarray scanning. The cross-talk ratio was calculated using a calibration slide. It was independent on the fluorophore concentration. However, the cross-talk ratio is different when using different kind of fluorophores, so it must be recalculated when changing fluorophores.

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