Improving FRET efficiency measurement in confocal microscopy imaging

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Spectral bleedthrough (SBT) ratio is dependent on the level of fluorescence intensity in confocal imaging. Precision Förster resonance energy transfer (FRET) algorithm corrects SBT ratio according to fluorescence intensity and avoids over- or under-estimation of SBT ratio. In this letter, we propose a new method to accurately measure the FRET efficiency of FRET plasmid in single living cells by combining the calculation of SBT in precision FRET algorithm with *E*-FRET formulae. We also use this method to measure the FRET efficiency of FRET-Bid, and find that in healthy A549 cells it is about 15%, which is verified by FRET acceptor photobleaching method.

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For dynamic living cell Förster resonance energy transfer (FRET) applications[1−3], the sensitized emission measurement is the most widely used approach[4−6]. Sensitized emission ($F_{\rm C}$) is defined as an increase in fluorescence emission from the acceptor due to FRET. With this method, spectral bleedthrough (SBT) correction is very crucial for the accurate determination of FRET efficiency (E). SBT mainly include the donor SBT (DSBT) resulting from the donor emission that is detected in the FRET channel, and the acceptor SBT (ASBT) that is caused by the direct excitation of the acceptor at the donor excitation wavelength.

Based on acceptor photobleaching method, Zal *et al.* proposed *E*-FRET formulae to convert $F_{\rm C}$ into E[7]:

$$E = \frac{F_{\rm C}/G}{F_{\rm C}/G + I_{\rm DD}}, \tag{1}$$

where G is a proportionality constant factor[4], $I_{\rm DD}$ is the fluorescence intensity of donor in donor channel with donor excitation, and $F_{\rm C}$ and G are calculated using[7]

$$F_{\rm C} = f - {\rm ASBT} - {\rm DSBT}, \tag{2}$$

$$G = \frac{F_{\rm C} - F_{\rm C}^{\rm post}}{I_{\rm DD}^{\rm post} - I_{\rm DD}}, \tag{3}$$

where $I_{\rm DD}^{\rm post}$ is the intensity of donor fluorescence in donor channel with donor excitation wavelength after incompletely photobleaching acceptor, and f represents the intensity in acceptor channel with donor excitation wavelength for double-labeled sample. $F_C^{\rm post}$, the sensitized emission after incompletely photobleaching acceptor, is obtained by Eq. (2).

G is constant for a given choice of fluorophores and imaging setup, and is independent of E[7]. G is generally determined using a control sample with FRET plasmid for the imaging setup and fluorophores. SBT correction is the most important factor for the accurate measurement of E from Eqs. (1)–(3).

Chen *et al.* have pointed out that SBT ratio is constant and independent of the fluorescence intensity level in wide-field microscopy system, but is not constant and is dependent on the fluorescence intensity level in confocal microscopy system[8]. Zal *et al.* validated their method using a SBT constant correction in wide-field microscopy systems but did not apply this method in confocal microscopy system[7]. Elangovan *et al.* proposed a precision FRET (PFRET) method to measure E, in which SBT calculation was dependent on the fluorescence intensity level[9,10]. Moreover, this algorithm can be used to estimate the SBT correction for the linear and nonlinear intensity distributions. However, PFRET requires measuring the detector quantum efficiencies at the donor and acceptor peak emission wavelengths respectively, and the transmission of filters in two channels.

In this letter, we measure the values of G and E from Eqs. (1)–(3), in which the SBT is obtained from PFRET algorithm. This method avoids considering the quantum efficiency of the detector, the transmission of filters, and the exposure time in comparison with PFRET algorithm[9,10]. Compared with *E*-FRET formulae[7], this method makes the calculation of SBT more accurate. We validate this method using FRET-Bid plasmid with known E measured by FRET acceptor photobleaching (Pb-FRET) technique as described in our previous work[11]. Pb-FRET, which has been verified by fluorescence lifetime imaging (FLIM), is used to measure the E as a comparison with that obtained by SBT ratio based on intensity image (IR) and constant SBT ratio (CR).

We performed our experiments on a Zeiss LSM 510 confocal microscope equipped with a 40× oil immersion objective (numerical aperture NA = 1.3)[12−15]. Our method was tested with measurements from A549 cells expressing CFP-Bax, YFP-Bcl-xl, and FRET-Bid. FRET-Bid consists of a donor (cyan fluorescent protein, CFP) and an acceptor (yellow fluorescent protein, YFP). Dual excitation imaging of three samples were performed

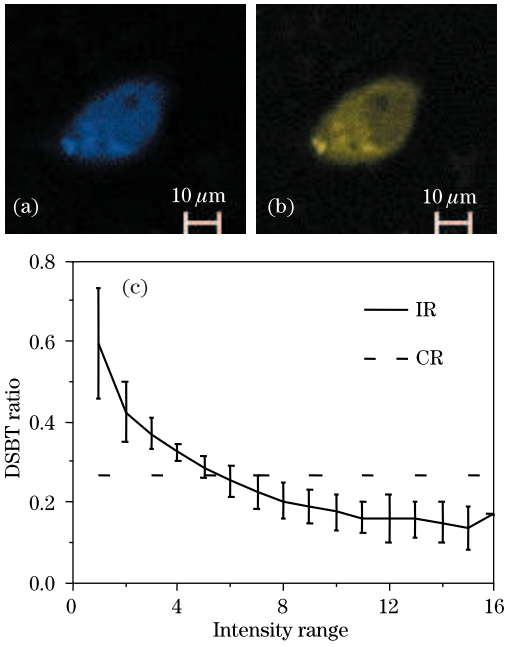

Fig. 1. Images of cells expressing CFP-Bax in (a) Ch_1 and (b) Ch_2 with 458-nm excitation; (c) DSBT ratios at different intensity ranges and constant DSBT ratio in our confocal microscopy system.

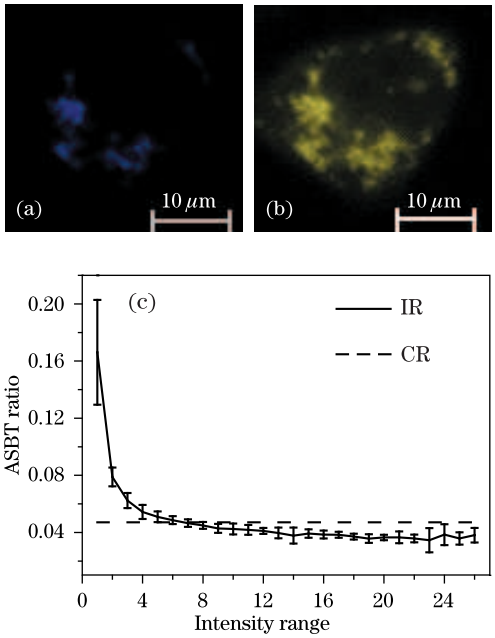

Fig. 2. Images of cells expressing YFP-Bcl-xl in Ch_2 with (a) 458-nm excitation and (b) 514-nm excitation; (c) ASBT ratios at different intensity ranges and constant ASBT ratio in our confocal microscopy system.

by alternating excitation wavelengths between 458 and 514 nm from an argon ion laser and collecting emission at 465–510 nm band-pass channel for CFP (Ch_1) and 520–555 nm band-pass channel for YFP (Ch_2). In our experiment, it is necessary to ensure the same conditions (gain, amplifier offset, laser intensity, pinhole, and scan speed) for living cells expressing donor (CFP) or acceptor (YFP) and both donor (CFP) and acceptor (YFP), and the SBT is the same in this case.

Figures 1(a) and (b) show the images of cells expressing CFP-Bax alone in Ch_1 and Ch_2. CFP is excited with 458-nm laser. According to the gray values of Ch_1, every 5 values are defined as a grade of the intensity range. Figure 1(c) shows the DSBT ratios at different intensity ranges. IR is the ratio of the fluorescence intensity between two channels at different intensity ranges and CR is the ratio of the fluorescence intensity between two channels.

The ASBT ratio was obtained using the same approach as DSBT ratio. Figures 2(a) and (b) show the images of cell expressing YFP-Bcl-xl alone in Ch_2 with alternative 458- and 514-nm laser excitation. Figure 2(c) exhibits the ASBT ratios of different ranges. Figures 1(c) and 2(c) show that IR is not constant at different intensity ranges, and the ratios of both ASBT and DSBT are very high particularly at low intensity levels. This may be due to the response of the spectral sensitivity of the photomultiplier tube (PMT)[8]. In this case, the CR and IR methods will provide different SBTs, resulting in different values of E. If we use CR method to correct SBT in our confocal system, we may underestimate the SBT. Therefore, the value of E obtained by IR method is lower than that by CR method according to E-FRET formulae.

Next, we used acceptor photobleaching to measure G. The acceptor (YFP) in the chosen region inside living cells expressing FRET-Bid was selectively incompletely bleached with the maximum of 514-nm laser line. Figures 3(a)–(f) show the images before and after incompletely photobleaching acceptor in single living cells expressing FRET-Bid. SBTs before and after incomplete acceptor photobleaching are also obtained using these images. The SBT results, obtained from SBT ratios[9,10], are substituted into Eqs. (2) and (3) to calculate the value of G.

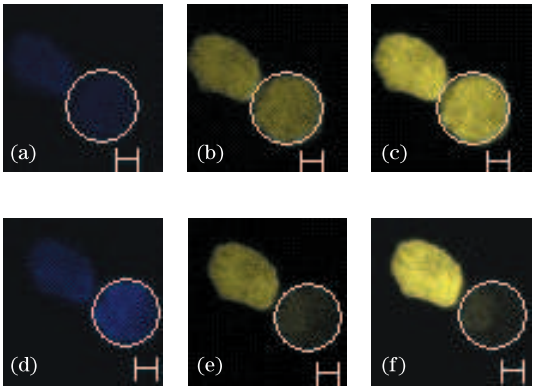

Fig. 3. Images of cells expressing FRET-Bid (a)–(c) before and (b)–(d) after photobleaching YFP. White circles indicate the photobleached cell. (a) and (d) In Ch_1 with 458-nm excitation; (b) and (e) in Ch_2 with 458-nm excitation; (c) and (f) in Ch_2 with 514-nm excitation. The scale bar is 10 μm.

Figure 4 shows the statistical results of the E of FRET-Bid in living cells from 10 cells using three methods. The E obtained from Eqs. (1)–(3) using SBT ratio correction based on IR is 15.1%±1.8%, which is consistent with 15.2%±2.9% obtained by using Pb-FRET technique[7], but is different from 21.2%±1.9% obtained using Eqs. (1)–(3) based on CR. We find that E by IR method is lower than that by CR method, resulting in a difference of 6% between these two approaches. Figures 1(c) and

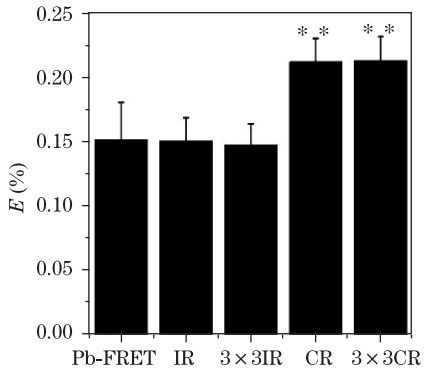

Fig. 4. Statistical results of the E of FRET-Bid by three FRET microscopy methods. Pb-FRET: FRET acceptor photobleaching method[8]; IR: Eqs. (1)–(3) using SBT ratio correction based on different intensity ranges; CR: Eqs. (1)–(3) using constant SBT ratio; 3×3 means the binning mode with the intensity average of 3×3 pixels. $^{**}P < 0.05$ compared with Pb-FRET.

2(c) clearly show that IR reduces gradually with the increase of intensity. Therefore, the SBT based intensity range is bigger than that based on CR, and the value of E obtained by IR method is lower than that by CR method according to E-FRET formulae.

In addition, 3×3 binning mode we used in Fig. 4 can reduce the effect of cell mobility on E. From the results shown in Fig. 4, we can find that the E obtained by 3×3 IR method is lower than that obtained by 1×1 IR method, but the E obtained by 3×3 CR method is slightly higher than that obtained by 1×1 CR method. The number of pixels with low fluorescence intensity in 3×3 binning mode is more than that in 1×1 binning mode. Therefore, the SBT obtained by 3×3 IR method is larger than that obtained by 1×1 IR method, so that the E by 3×3 IR method is lower than that by 1×1 IR method according to E-FRET formulae. Similarly, SBT obtained by using 3×3 CR method is slightly lower than that obtained by using 1×1 CR method, leading to the slight higher E by 3×3 CR method.

These results clearly demonstrate that it is more accurate to measure E using IR method in confocal microscopy.

In conclusion, we propose a new method to obtain E by combining the calculation of SBT in PFRET algorithm with E-FRET formulae, which is effective to remove the SBT contamination in FRET signals.

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