Emerging Techniques in Modern Microscopies

MIM.2.P027
Spectral “bleed-through” correction makes possible FRET microscopy of intracellular organelles

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Keywords: FRET, spectral, bleed-through

Quantitative Forster resonance energy transfer (FRET) microscopy provides a mean for studying protein interactions in cells. FRET microscopy has unavoidable spectral “bleed-through”. Spectral cross-talk or bleed-through (SBT) is an acronym for appearance of unwanted signal in a microscopy image as result of excitation/emission spectra overlaps between different fluorescence markers. Common wisdom recommends carefully choose fluorescent markers combination, emission filters and use sequential excitation to avoid SBT effect. Unfortunately in FRET microscopy spectral cross-talk is inevitable, since the FRET signal and fluorescence of directly excited acceptor have the same spectrum (for review FRET principle and technology see, for example, Jares-Erijman & Jovin, 2003; Berney & Danuser, 2003; Jares-Erijman & Jovin, 2006; Ishikawa-Ankerhold et al, 2012; Preus & Wilhelmsson, 2012, Knox, 2012). In the last 15 years multiple methods were developed to handle FRET for spectral cross-talk. They include indirect methods, like acceptor photo-bleaching [Wouters et al, 1998; Gu et al., 2004] as well as direct corrections of SBT [Cordon et al., 1998; van Rheenen et, al, 2004; Wallrabe et al., 2006]. It was shown that correction provides reliable result in relatively large fluorescent areas with relatively high ratio of FRET signal to SBT. When either area of interest becomes small or ratio of donor/acceptor is far from unity, the correction becomes unstable and results even in negative FRET signal. This effect makes impossible direct FRET microscopy of single intracellular organelles, like endosomes or secretory granules. Moreover, it was shown that intensity of donors influence the measured FRET efficiency even after correction [Wallrabe, 2006].

The most commonly used FRET correction of SBT [Cordon et al, 1998] includes 9 images, which comes from 3 combinations of excitation/emission (Table I) and 3 combination of sample staining (Table II).

In these tables only second lines correspond to FRET measurement *per se*. Other combinations are used for calculation the SBT correction coefficients. In other word for proper direct FRET measurement researcher has to make eight calibration images and one FRET image. Since all staining combination impossible to have on the same sample, in addition to FRET sample (line # 2, Table II), other two samples are used for SBT coefficient calibration. In sake of simplicity we consider the major SBT contribution, which corresponds to direct excitation of acceptor by donor-exciting wavelength. This contribution could not be suppressed by emission filter selection and, unfortunately, is not much suppressible by excitation laser selection for most commonly used FRET donor/acceptor couples and commercially available microscopes. Another SBT of red wing of donor through FRET emission filter could be minimized in expense of some decrease of method sensitivity.

First, we revisit correction of SBT in the control conditions where we can directly separate real signal of interest from SBT. For this we took images of endosomes labeled with EEA1 antibodies conjugated to Alexa555 and follow pulse of Alexa488 labeled LDL. The two channel imaging was done with exciting lasers 488 and 561 sequentially. Set of emission filters was chosen to allow significant bleed-through from Alexa-555 excited by 488nm laser line to the 488 channel (Fig.1). As control we have repeated the same experiment without EEA1-Alexa555 staining. Endosomes were found on both channels independently as described before (Rink et al, 2005). Classical SBT correction procedure [Cordon et al., 1998] despite gave correct estimation of mean intensity of endosomes, but for many individual endosomes predicted negative signal in intensity-of-interest channel.

In this work we revisited the mechanism of spectral bleed-through image formation and demonstrate that source of correction failure for individual endosomes is intrinsic Poisson noise of fluorescence microscopy. We develop new spectral bleed-through correction formulas, which a) properly corrects bleed-through that was confirmed by control experiment without EEA1-Alexa555 staining, b) produces only positive estimation of intensities-of-interest (Fig.2C).

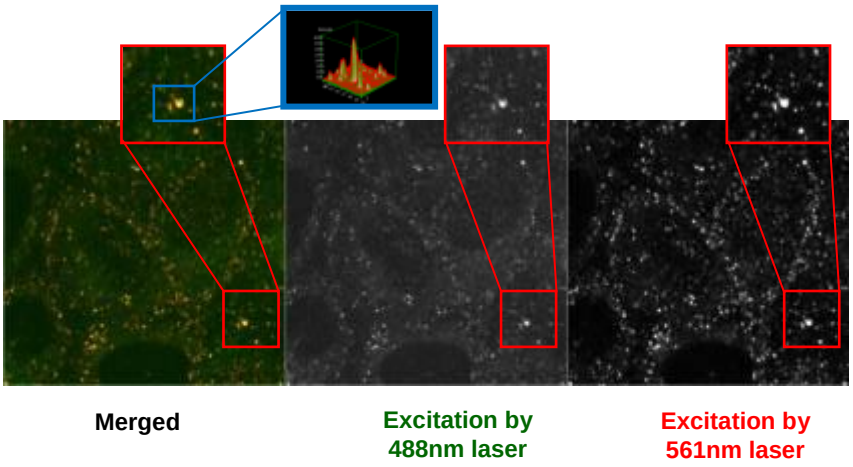

Figure 1. Control images: HeLa cells stained only by APPL1-Alexa555 antibodies. Images were obtained by Zeiss 510 confocal microscope with individual gain selection and separate excitation by 561 and 488 lasers. Individual endosomes were recognized and quantified by image intensity fitting as described before (Rink et al, 2005).

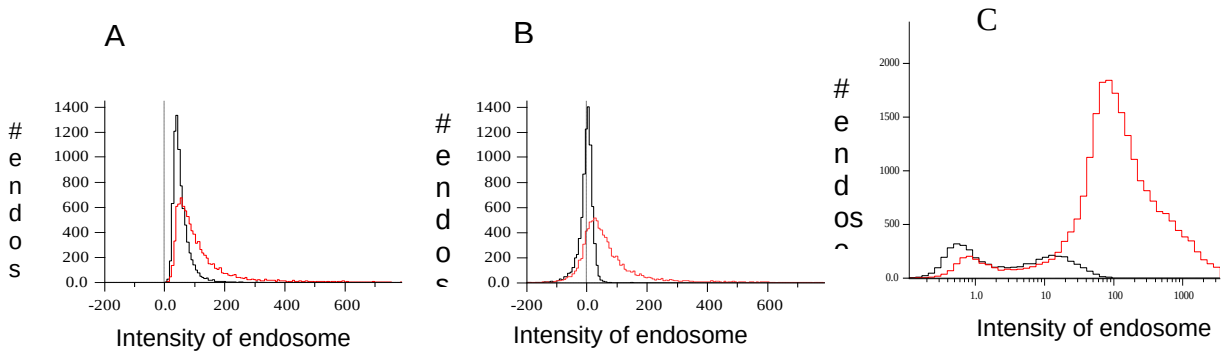

Figure 2. Control in absence of A-488 labeled marker (SBT only) – black. LDL-A-488 uptake for 5 min (real signal + SBT) - red. A. Non-corrected endosome intensity (integral intensity of endosome divided by endosome area) distribution. B. Corrected endosome intensity distribution with constant SBT coefficient.
C. Corrected endosome intensity distribution with probabilistic SBT estimation

Table I

Excitation	Emission
Donor	Donor
Donor	*Acceptor*
Acceptor	Acceptor

Table II

Donor Staining	Acceptor Staining
+	-
+	+
-	+