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Spectral measurement of acceptor-to-donor extinction coefficient ratio in living cells

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ABSTRACT

This report presents a simple method named as sp-ECR to determine the molar extinction coefficient ratio ($\gamma(\lambda_{ex})$) of acceptor-to-donor in living cells at excitation wavelength λ_{ex}, which is closely associated with the acceptor cross-excitation, the hardest issue of FRET quantification. sp-ECR determines $\gamma(\lambda_{ex})$ by spectrally unmixing the emission spectrum of a donor–acceptor tandem construct under λ_{ex} excitation without any additional references, such that this method can be performed under optimal imaging condition. We used sp-ECR to measure the $\gamma(458)$ of Venus/Cerulean in living HepG2 cells on a confocal microscope, and the measured values were consistent with those obtained by lux-FRET method. We also used sp-ECR to measure the $\gamma(458)$ values of Venus/Cerulean and YFP/CFP as well as YFP/GFP, the commonly used FRET FPs pairs in other two kinds of cancer cell lines on the confocal microscope, and found that the extinction coefficients of FPs depended on cell lines. After predetermining the $\gamma(458)$ of Venus to ECFP, we used sp-ECR method to monitor the staurosporine (STS)-induced dynamical caspase-3 activation in single live A549 cells expressing SCAT3 by spectrally resolving the absolute FRET efficiency of SCAT3, and found that STS-induced caspase-3 activation in single cells is a very rapid process within 20 min.

1. Introduction

Fluorescent proteins (FPs)-based fluorescence resonance energy transfer (FRET) has become a powerful tool for quantitative analysis of protein–protein interaction, protease and kinase activities in living cells (Heim and Tsien, 1996; Düssmann et al., 2010). However, spectral bleed-through (donor fluorophore emission into the acceptor channel) and acceptor cross-excitation (direct excitation of acceptor fluorophore by donor excitation) contaminate the FRET signals, making accurate FRET quantification very difficult in current donor–acceptor FP pairs such as ECFP/Cerulean-EYFP/Venus, GFP-EYFP/Venus and GFP/YFP-mCherry/mRed (Gordon et al., 1998; Xia and Liu, 2001; Elder et al., 2009).

Hence complicated crosstalk correction is essential for quantitative FRET measurement (Elder et al., 2009; Wang et al., 2010). Donor emission spectral bleedthrough can be readily separated by directly dividing the emission spectra of FRET sample into contributions from the donor and acceptor based upon the spectra of a donor only and acceptor only sample (Thaler et al., 2005; Chen et al., 2007; Levy et al., 2011; Mutafa et al., 2013). However, the acceptor cross-excitation signal cannot be directly distinguished from the sensitized acceptor signal due to the same spectra of both FRET signal and cross-excitation signal (Zimmermann, 2005; Thaler and Vogel, 2006; Mutafa et al., 2013). An additional acceptor-only reference is usually utilized to obtain a cross-excitation correction factor that depends on the donor and acceptor excitation intensities, system settings and the acceptor-to-donor extinction coefficient ratio (Chen et al., 2007; Megías et al., 2009; Sun et al., 2009; Levy et al., 2011). In principle, the acceptor cross-excitation fraction is intrinsic to the extinction coefficient ratio of acceptor to donor and their relative concentrations as well as the optic properties of instrument used.

In realty, the exact excitation wavelength depends on the excitation light source and the transmission property of instrument used. Moreover, many publications have shown that the

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extinction coefficients of FPs vary in different cell lines (Lakowicz, 1999; Wlodarczyk et al., 2008). Therefore, the extinction coefficient ratio of acceptor to donor in living cells cannot be from publications and must be measured on a particular instrument. Wlodarczyk and co-workers proposed a FRET quantification method named as lux-FRET that linearly resolves two emission spectra of FRET sample at two different excitation wavelengths (Wlodarczyk et al., 2008). In fact, lux-FRET also can be utilized to determine the extinction coefficient ratio ($\gamma(\lambda_{ex})$) of acceptor to donor in living cells as following (Wlodarczyk et al., 2008):

$$\gamma(\lambda_{ex}^{i}) = \frac{\varepsilon_A(\lambda_{ex}^{i})}{\varepsilon_D(\lambda_{ex}^{i})} = \frac{1}{r^{ex,i}} \frac{[D^{ref}]}{[A^{ref}]}, \quad (1)$$

where $[D^{ref}]$ and $[A^{ref}]$ are the concentrations of donor (D)-only and acceptor (A)-only reference samples, respectively; and $r^{ex,i}$, the excitation ratio at the i excitation wavelength, can be measured as:

$$r^{ex,i} = \frac{F_D^{i,ref}(\lambda)\varphi_A e_A(\lambda)}{F_A^{i,ref}(\lambda)\varphi_D e_D(\lambda)}, \quad (2)$$

where $F_D^{i,ref}$ and $F_A^{i,ref}$ are the spectral intensities of D-only and A-only references at the i excitation wavelength, and the fluorescence intensity of a particular fluorophore depends on many parameters intrinsic to the fluorophore and the instrument used to detect it; φ_D and φ_A are quantum yields of donor and acceptor; $e_D(\lambda)$ and $e_A(\lambda)$ are standard emission spectra of the donor and acceptor fluorophores normalized to unit area. The spectra (F^i) of a tandem donor–acceptor construct at the i excitation wavelength was unmixed according to $F_D^{i,ref}$ and $F_A^{i,ref}$ as follow:

$$F^i = \delta^i F_D^{i,ref} + \alpha^i F_A^{i,ref} \quad (i = 1 \text{ and } 2) \quad (3)$$

The apparent relative concentrations of donor (δ^i) and acceptor (α^i) can be obtained from Eq. (3), and the concentration ratio $[D^{ref}]/[A^{ref}]$ between donor and acceptor references is (Wlodarczyk et al., 2008):

$$\frac{[D^{ref}]}{[A^{ref}]} = \frac{1}{R_C} \frac{\alpha^1 r^{ex,2} - \alpha^2 r^{ex,1}}{(r^{ex,2} - r^{ex,1})\delta^1 + \alpha^2 - \alpha^1}, \quad (4)$$

where R_C is the acceptor/donor stoichiometry of the tandem construct. In this procedure, we must simultaneously prepare three live-cell samples separately expressing D-only, A-only and D–A tandem construct, and all detections for the three kinds of samples must be performed under constant imaging conditions.

In this report, we developed a novel and simple spectral unmixing method for the measurement of $\gamma(\lambda_{ex})$ in living cells. This method named as sp-ECR can quickly determine the $\gamma(\lambda_{ex})$ in living cells by linearly unmixing the emission spectrum of a donor–acceptor tandem construct under λ_{ex} excitation without any additional references. Therefore, sp-ECR can be performed under the optimal imaging conditions. We used both sp-ECR and lux-FRET methods to measure the $\gamma(458)$ values of Venus-to-Cerulean, the commonly used FPs pairs, in the same living cells to validate sp-ECR method on a confocal fluorescence microscope.

2. Materials and methods

2.1. Reagent and plasmids

Turbofect™ *in vitro* transfection reagent was purchased from Fermentas (USA). Cerulean and Venus-Kras plasmid purchase from Addgene Company. The standard FRET constructs including C32V (Cerulean-32-Venus, Addgene plasmid 29396) and VCV (Venus-5-Cerulean-5-Venus, Addgene plasmid 27788) were kindly provided by the Vogel lab (National Institutes of Health, Bethesda, MD, USA). Plasmid DNA of 18AA was kindly supplied by professor Kaminski (Elder et al., 2009), plasmid DNA of GFP-YFP was kindly supplied by professor Houtsmuller (Dinant et al., 2008), and plasmid DNA of SCAT3 was kindly supplied by professor Masayuki (Takemoto et al., 2003). Staurosporine (STS) was purchased from Sigma–Aldrich Co. LLC (Santa Clara, USA).

2.2. Cell culture and transfection

A549 and HepG2 cell lines were obtained from the Department of Medicine, Jinan University (Guangzhou, China), DU145 cell line was obtained from Laboratory Animal Centre, Sun Yat-Sen University (Guangzhou, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, USA) supplemented with 10% foetal calf serum (FCS, Sijiqing company, Hangzhou, China) in 5% CO_2 at 37 °C in a humidified incubator. For transfection, cells were cultured in DMEM supplemented with 10% serum at a density of 4×10^4 cells/ml in 35-mm glass dish. After 24 h, when the cells reached 70–90% confluence in DMEM containing 10% FBS at 37 °C in 5% CO_2, plasmid DNA of these plasmids were transfected into the cells by using Turbofect™ *in vitro* transfection reagent in 35-mm dish for 24–48 h.

2.3. Microscope platform

Spectral imaging experiments were performed on a confocal scanning microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany) with a 40×/NA 1.3 oil immersion objective. For our experiments, FPs were excited by 458-nm line from an argon laser, a 458 dichroic mirror was present in the excitation path and emission spectra were sampled in the range of 464.8–603.9 nm (14 spectral bins) with 10.7 nm spectral resolution. For lux-FRET experiments, the spectral images of donor and acceptor as well as tandem construct were collected in the range of 464.8–603.9 nm for 458 nm excitation with 458 dichroic mirror and in the range of 496.6–603.9 nm for 488 nm excitation with 405/488 dichroic mirror.

2.4. Partial acceptor photobleaching-based FRET measurement (PbFRET measurement)

Partial acceptor photobleaching-based FRET quantification in living cells was also performed on a confocal microscope system with a 40×/NA 1.3 oil objective (LSM510 Meta, Carl Zeiss, Oberkochen, Germany) as described previously (Yu et al., 2012). The excitation wavelength was 458 nm for donor and 514 nm for acceptor. The acceptor in the chosen region inside living cells was selectively bleached with the maximum of 514 nm laser line. Donor channel was 475–496 nm, acceptor channel was LP 560 nm. The FRET efficiency of 1D–*n*A construct was calculated as following (Elder et al., 2009; Yu et al., 2012):

$$E = \frac{1 - I_{DD}/I_{DD}^{AP}}{1 - (1 - x/n)I_{DD}/I_{DD}^{AP}}, \quad (5)$$

where I_{DD} is the fluorescence intensity detected with donor channel that selectively collect donor fluorescence at donor excitation before (I_{DD}) and after (I_{DD}^{AP}) partial acceptor photobleaching, and x is the degree of acceptor photobleaching.

2.5. Statistical analysis

Results are expressed as mean ± SD. Data were analyzed by repeated-measures ANOVA with parametric methods and LSD multiple comparison using the statistical software SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Throughout the work, P values less than 0.05 were considered to be statistically significant.