Chapter 13

Fluorescence Resonance Energy Transfer Microscopy for Measuring Chromatin Complex Structure and Dynamics

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Abstract

The Polycomb group (PcG) proteins form regulatory complexes that modify the chromatin structure and silence their target genes. Recent works have found that the composition of Polycomb complexes is highly dynamic. Defining the different protein components of each complex is fundamental for better understanding their biological functions. Fluorescent resonance energy transfer (FRET) is a powerful tool to measure protein–protein interactions, in nanometer order and in their native cellular environment. Here we describe the preparation and execution of a typical FRET experiment using CFP-tagged protein as donor and YFP-tagged protein as acceptor. We further show that FRET can be used in a competition assay to measure binding affinities of different components of the same chromatin complex.

Key words Fluorescence resonance energy transfer (FRET), Acceptor photobleaching, CFP, YFP, Confocal microscopy, PcG

1 Introduction

The Polycomb Group (PcG) proteins form large multimeric complexes that directly interact with the chromatin at their target genes [1]. Previous studies based on biochemical or X-ray crystallography analysis have identified two main families of PcG complexes with a substantial variety in number and composition of PcG proteins [2–6] (Fig. 1). However these tools do not permit to analyze whether the different complex compositions are independent entities with separate function. In addition these biochemical approaches do not allow measuring those dynamic changes of PcG composition that may occur in response to different environmental stimuli.

A common technique used to monitor protein–protein interactions in live or fixed cells is fluorescence resonance energy transfer (FRET) [7]. FRET measurement is based on energy transfer from a donor fluorophore to a suitable acceptor fluorophore through a long-range dipole–dipole coupling mechanism. FRET occurs only when the distance separating the two fluorophores is less than

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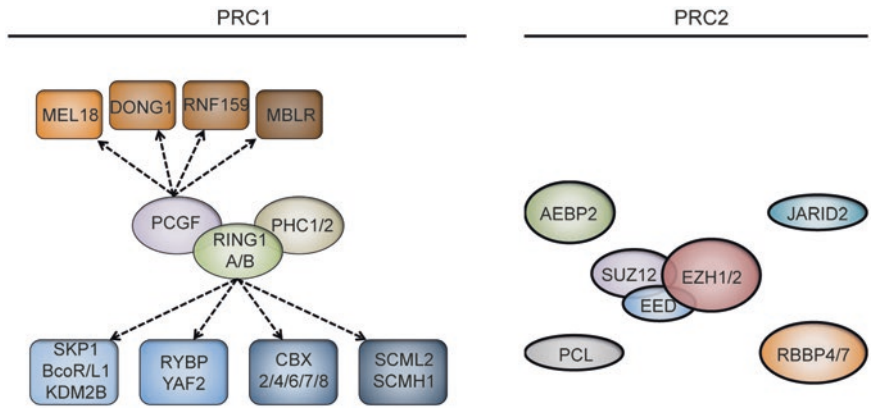

Fig. 1 Composition of the main Polycomb complexes. A schematic representation of the core components of PRC1 and PRC2 are shown. The diversity of Polycomb complexes is shown through the incorporation of homologous proteins. In the PRC1 complex, the core subunits include RING1A/B and a member of both CBX and HPH families. The core subunits of PRC2 are EZH1/2, EED, and SUZ12 proteins. Depending on different environmental stimuli or cell type, additional protein components can associate with the PRC1 and PRC2 complexes, respectively

approximately 10 nm because the efficiency of this energy transfer decreases proportionally to the sixth power of the distance separating the two fluorophores (Fig. 2a, b) [8, 9]. In addition, FRET takes place only if the donor emission spectra overlap substantially with the acceptor absorption spectra (Fig. 2c) [10]. These features, combined with recent technological advances in light microscopy imaging and an increasing number of different fluorescent proteins, make FRET a sensitive tool necessary to obtain spatial and temporal distribution of protein association within the cellular context.

In addition FRET assay can be used to study competition between two proteins for binding to the same protein. This FRET-based competition assay has been previously used to characterize interaction of inhibitors with kinases [11, 12]. In this method, an unlabeled competitor may perturb the dynamic interaction frequencies between donor-labeled and acceptor-labeled proteins, reducing the FRET signals. This is because the competitor displaces the acceptor-labeled protein, separating the two fluorophores, which can no longer transfer energy to each other (Fig. 3). This FRET-based competition assay has several advantages including high sensitivity and reproducibility, and it is compatible with high-throughput studies, thereby making it a potential tool to screen compounds against therapeutic targets [13–15].

Herein, we give a detailed description of FRET microscopy imaging and data analysis. The first part of this protocol describes the sample preparation for FRET microscopy experiments of fixed NIH-3T3 cells, which can be similarly used for analyses on living cells. In the second part we describe the experimental setup to optimize the image acquisition. This is followed by a description of

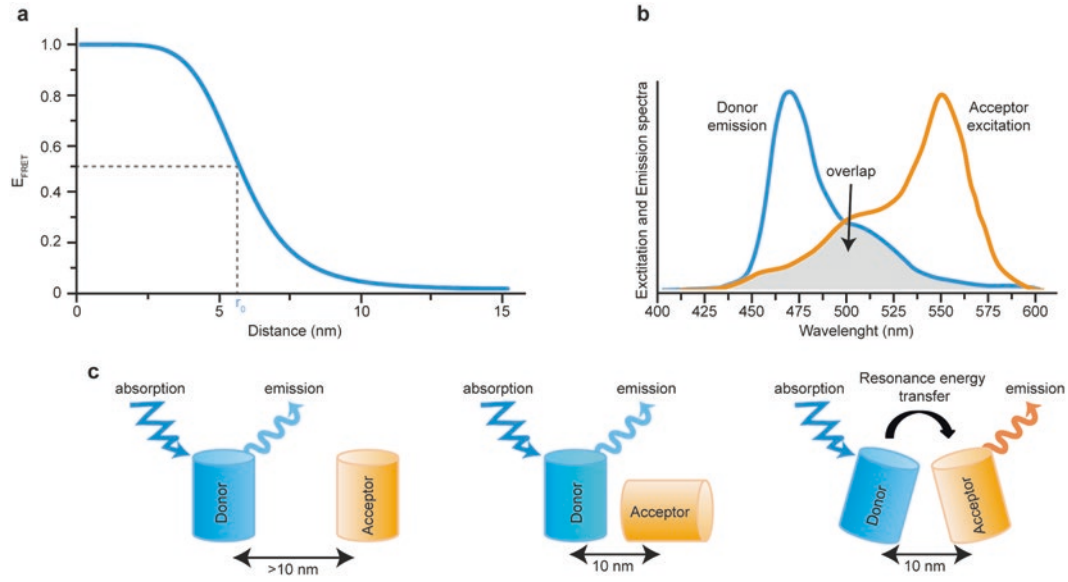

Fig. 2 Principles of fluorescence resonance energy transfer (FRET). (**a**) Diagram of the FRET efficiency as a function of the distance between a donor and an acceptor fluorophore (R_0). (**b**) FRET occurs only when the donor emission spectrum overlaps with the acceptor excitation spectrum. The *grey area* corresponds to the overlapped region. (**c**) Schematic representation of FRET. When the FRET donor and acceptor are more than 10 nm of one another (*left*) or are not correctly oriented (*middle*), then no FRET occurs and the donor emits fluorescence. If the donor and acceptor are within 2–10 nm of one another and correct oriented (*right*), excited donor transfer its energy by a nonradioactive process to the acceptor, causing it to emit fluorescence

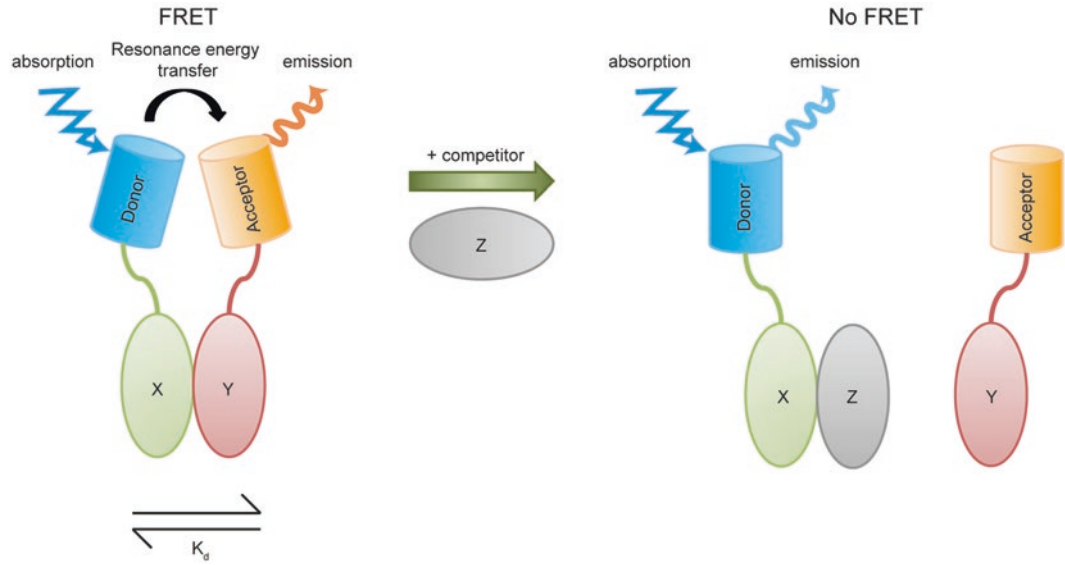

Fig. 3 Schematic representation of FRET-based competition assay. The scheme represents the basic principle of FRET competition assay. When an unlabeled target protein (Z) competes with the acceptor-labeled protein (Y) for binding with donor-labeled protein (X), the FRET efficiency may decrease or not occur

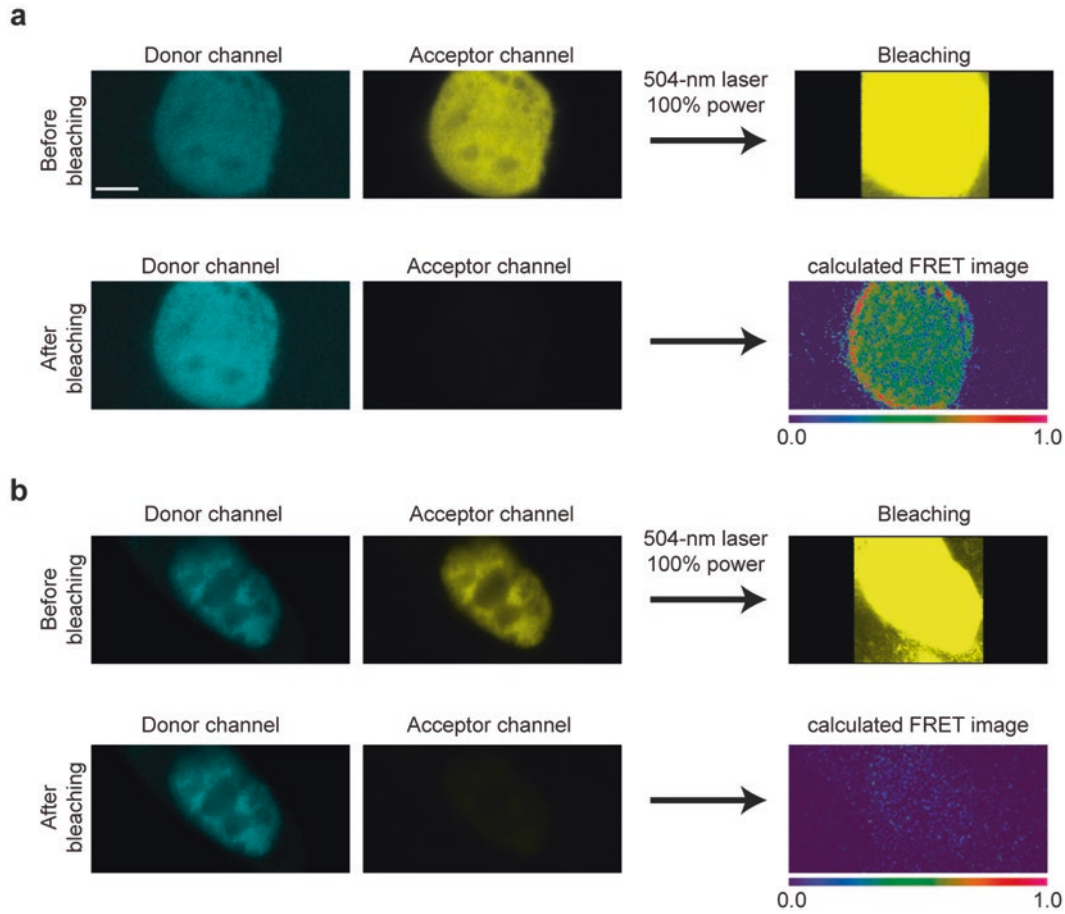

Fig. 4 An example of FRET AB microscopy images. This figure shows donor (CFP) and acceptor (YFP) fluorescent intensity images (before and after bleaching). Bleaching is performed using 514-nm laser line with the 100% of power for four bleach iterations. FRET efficiency is calculated according to equation described in Subheading 3.3, **step 7** and is presented as a color-coded map of FRET intensity. (**a**) FRET analysis of CFP fused to YFP (positive control) transiently expressed in NIH/3T3 cells. (**b**) FRET analysis of CFP-EED mixed with unconjugated YFP (negative control) transiently expressed in NIH/3T3 cells. Scale bar equals 5 μm

data analysis procedures for the determination of FRET efficiency (Fig. 4). In the last part, we describe methodologies to perform FRET-based competition analyses.

2 Materials

1. Tissue culture dishes, six-well.
2. Cover glass: 25 mm diameter.
3. 0.1% v/v gelatin solution in PBS. Keep at 4 °C.
4. NIH/3T3 cells.

5. Growth medium: DMEM supplemented with 10% v/v FBS, 1× L-glutamine, 1× nonessential amino acids, and 1× penicillin/streptomycin. The medium can be stored for up to 1 month at 4 °C.
6. Plasmid vectors: CFP-protein#1 and YFP-protein#2 (to test for FRET between protein#1 and protein#2), CFP–YFP fusion, unconjugated CFP and unconjugated YFP.
7. Lipofectamine 2000.
8. 4% paraformaldehyde solution. Filter the solution through a 0.45 μm filter prior to use.
9. Forceps.
10. Leica TCS SP5 confocal microscope.
11. 63×/1.4 NA oil immersion objective.
12. Coverslip holder: Autofluor cell chamber.
13. Leica SP5 software.

3 Methods

3.1 Cell Culture Transfection

1. Plate NIH-3T3 cells 24 h prior to transfection: remove growth medium from a 100 mm cell culture dish and rinse cells once with 5 ml of PBS, swirling gently (*see* **Note 1**).
2. Add 2 ml of 0.05% trypsin–EDTA to the cells and incubate at 37 °C for 2–3 min or until cells are detached from the culture dish, then add 4 ml of growth medium to neutralize trypsin digestion.
3. Count and seed 600,000 cells into a single well of a six-well plate containing a gelatin-coated coverslip, for each transfection condition.
4. Prepare the transfection mix using a ratio of 2.5 μl of Lipofectamine 2000/1 μg of total DNA. Transfect the cells with 4 μg of total DNA with the following constructs: (a) CFP fused to YFP (positive control, *see* **Note 2**); (b) CFP-protein#1; (c) YFP-protein#2; (d) CFP-protein#1, mixed with unconjugated YFP, and YFP-protein#2, mixed with unconjugated CFP (negative control, *see* **Note 3**); (e) CFP-protein#1 mixed with YFP-protein#2 (*see* **Notes 4**–**7**). For each transfection, the DNA and Lipofectamine 2000 are separately diluted into 500 μl of Opti-MEM in a 1.5 ml plastic micro tube, then combine Lipofectamine 2000 with DNA and mix it by vortexing.
5. Incubate the mixture for 20 min at room temperature.
6. Remove the growth medium from each well and wash gently with Opti-MEM medium. Add gently DNA–Lipofectamine 2000 mixture to each well (*see* **Note 8**). Add 1 ml of growth medium after 4 h and incubate the cells at 37 °C for 24 h.

7. Fix the cells by adding 4% paraformaldehyde and incubate for 20 min at room temperature. Wash the coverslips three times with PBS. The samples can be stored at 4 °C in the dark for 2 weeks in PBS before continuing (*see* **Note 9**).

3.2 Experimental Conditions Setting

1. Prepare the TCS SP5 confocal microscope (or comparable system) with a 40-mW argon laser set at 30% efficiency and tuned to lines at 458 and 514 nm.
2. Select FRET AB tool of SP5 software (Leica) and configure the acquisition parameters to 8-bit images, 512×512 pixels such that the region for FRET measurement occupies ~70% of the image, sequential scanning and pinhole to 1 Airy unit.
3. Remove the coverslip from the wells using forceps and place it into coverslip holder. Fill the holder with PBS.
4. Select a high-resolution immersion objective, such as 63×/1.4 NA, and apply immersion medium.
5. Start by acquiring cells containing only CFP-tagged protein. Find some fluorescent cells using microscope ocular and center them in the middle of the field of view.
6. Switch to the confocal mode, then adjust the laser transmission at 30% power for 458-nm laser and collect CFP emission from 465 to 495 nm. Set the image acquisition parameters to a maximum offset of 20, detector gain of 750, digital gain of 1 and line averaging of 4. Acquire an image of the CFP emission from a single cell and save the imaging parameters (*see* **Note 10**).
7. Repeat Subheading 3.2, **steps 5 and 6** with cells containing only YFP-tagged protein. Set the laser transmission at 30% power for 514-nm laser line and collect YFP emission from 555 to 630 nm (*see* **Note 11**).
8. Save the adjusted imaging parameters and do not change them in subsequent analyses.

3.3 Perform the Acceptor Photobleaching FRET Analysis

FRET Acceptor photobleaching involves measuring of the variation of the donor fluorescence in the presence of an acceptor. If FRET occurs, you will measure an increase of donor fluorescent intensity after photobleaching of the acceptor, as shown in Fig. 4.

1. Open the Edit Bleach window on the TCS SP5 to define the bleaching conditions.
2. Set up bleaching with a 514-nm laser line with the 100% of power (*see* **Note 12**).
3. Define the region of interest (ROI) and plot the ROI into the cell image where the photobleaching should occur (*see* **Note 13**).
4. To enable sufficient bleaching of the YFP-tagged protein, set the number of bleaching iteration at 4.

5. Run the bleaching experiment. The software acquires five images in a precise sequence: images of donor and acceptor before bleaching, followed by the bleaching of the acceptor and images of donor and acceptor after bleaching (Fig. 4).
6. The result in the ROI is automatically displayed and corresponds to your entire bleached area (Fig. 4). If necessary, it is possible to select additional ROI for better interpretation of results.
7. Apparent energy transfer efficiency is calculated as follow:

$$\mathrm{FRET}_{\mathrm{eff}} = \frac{D_{\mathrm{post}} - D_{\mathrm{pre}}}{D_{\mathrm{post}}}$$

   Here, D_{post} and D_{pre} refer to the intensity of the donor in a region of interest before and after the selective photobleaching of the acceptor. The $\mathrm{FRET}_{\mathrm{eff}}$ is considered positive when $D_{\mathrm{post}} > D_{\mathrm{pre}}$.
8. Repeat Subheading 3.3, **steps** from **3** to **6** for at least 30 individual cells.

3.4 Perform the Acceptor Photobleaching FRET Competition Analysis

In order to analyze whether two proteins or two isoforms of the same protein may compete for interaction with the protein of interest, a FRET competition analysis can be performed. In this method, FRET donor is bound to FRET acceptor and if the two fluorophores are in proximity with the proper orientation, FRET will occur between them. Displacement of acceptor with an unlabeled competitor can reduce FRET signal, indicating that the two proteins compete for the binding with FRET donor. In order to evaluate the FRET competition results, is appropriate to use different molar ratio between acceptor-labeled protein and the unlabeled competitor (e.g., 1:1, 1:4, and 1:8). In the case in which the investigated protein compete for their association with the protein of interest, the FRET efficiency should decrease proportionally to the increment of the acceptor–competitor ratio.

1. Transfect the cells with 4 μg of total DNA with the following constructs: (a) CFP fused to YFP (positive control); (b) CFP-protein#1, mixed with unconjugated YFP, and YFP-protein#2, mixed with unconjugated CFP (negative control); (c) CFP-protein#1 mixed with YFP-protein#2 and unconjugated protein#3 using different ratios; (d) CFP-protein#1 mixed with YFP-protein#3 and unconjugated protein#2 using different ratios (*see* **Note 14**).
2. Repeat steps from Subheading 3.2 to Subheading 3.3, to measure FRET efficiency. In case of competition between protein#2 and protein#3 for interaction with protein#1, reduction of FRET efficiency respect to the steady state will be measured.

4 Notes

1. This protocol is optimized for fixed NIH/3T3 cells. With appropriate modifications (e.g., coating coverslips, cell number, and quantity of transfected DNA), it may be used for other cell lines or using living cells. In the latter case, it would be necessary to keep cells within an humidified chamber with controlled temperature, thus avoiding perturbation of the cellular conditions.
2. It is appropriate to examine the setting and the maximum FRET efficiency using the positive control containing the two fluorophores connected with a short peptide linker (CFP–YFP).
3. It is appropriate to analyze the negative control: CFP-protein#1, mixed with unconjugated YFP, and YFP-protein#2, mixed with unconjugated CFP, which should not give FRET. This excludes that the fluorescent proteins affect the interaction.
4. Donor–acceptor choice is crucial for FRET. In fact, the FRET AB requires that the donor is a stable fluorescent protein, while the acceptor can be easily bleached during experimental conditions. Moreover, FRET efficiency dependent on both the distance between donor and acceptor molecules, and also on the overlaps between the donor molecule emission and acceptor molecule excitation spectra. The described protocol is optimized for CFP and YFP fluorescent proteins; however, other donor–acceptor pairs follow the same criteria (e.g., Cy3-Cy5 or BFP-GFP).
5. Before proceeding with FRET analysis it is appropriate to find the correct conditions of transfection since the ratio between the donor and acceptor strongly influences the outcome of FRET assays. Define experimental conditions in which the CFP and YFP-fusion protein are expressed at approximately equal amounts by performing immunoblot analyses of the transfected cells. Once you have optimized your experimental conditions, perform FRET assay putting particular attention on analyzing those cells which are expressing similar level of the two fluorescent protein, by comparing their relative intensity to the emission of the CFP–YFP positive control.
6. Certain proteins might cluster into aggregates making fluorescent dots that strongly affect the FRET analyses. In these cases, it would be necessary to tune the level of expression of the protein of interest and/or to co-transfect non-conjugated components of the investigated complex, thereby facilitating the proper organization of soluble form of the chromatin complex.

7. The effect of the N- or C-terminal fusion of the fluorescent protein to the protein of interest in terms of its localization and FRET efficiency should be carefully evaluated. Specifically, the tagged fluorescent proteins may cause steric effects and/or changing protein complex organization.
8. It is appropriate to work gently in order to avoid detachment of the cells from coverslips.
9. Even if the samples can be stored at 4 °C in the dark for 2 weeks, it is necessary to perform the analyses as soon as possible in order to avoid a reduction in the fluorescence intensity. Alternatively, after cell fixation the coverslips can be mount on a microscope slides and stored at −20 °C.
10. To exclude that CFP excitation can interfere with the excitement of YFP, excite the CFP and acquire an image in both the CFP and YFP detection channels. In case of high background signal in YFP image, adjust the setting for the CFP excitation.
11. To exclude that YFP excitation can interfere with the excitement of CFP, excite the YFP and acquire an image in both the CFP and YFP detection channels. In case of high background signal in CFP image, adjust the setting for the YFP excitation.
12. In case the donor will result bleached, decrease the power of the 514-nm laser reducing the strength of the photobleaching. Otherwise, decrease the scan speed or reduce the number of bleaching iterations.
13. If FRET takes place in a precise region of the cell, the ROI can be drawn on that area. However, in order to obtain reliable measures of intensity another ROI should be drawn in another compartment of the analyzed cell and the retrieved intensity should be use to determine the relative background signal.
14. Even for this experimental condition, test the proper ratio between the donor, acceptor, and competitor. In order to maintain the ratio of transfection, replace unconjugated competitor with empty vector in steady state condition.

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