FRET-based Quantification of Protein-Protein Interactions using the Opera Phenix High Content Screening System

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1 Abstract

FRET-based imaging assays are widely utilized to study protein-protein interactions or signal transduction processes in living cells. Here, we present a high content imaging assay to study the interaction between the pro-apoptotic protein Bad and the anti-apoptotic protein Bcl-XL in living MCF7 cells. In our assay, Bad was fused to the fluorescent protein Venus serving as the FRET acceptor while Bcl-XL was fused to mCerulean3 acting as the FRET donor.

In high content screening assays, FRET is commonly measured using the sensitized emission method, which requires sequential acquisition of the donor image and the FRET image (donor excitation, acceptor emission). This sequential approach reduces the acquisition speed with conventional high content imaging instrumentation.

In the study presented here, we take advantage of the 4 sCMOS cameras in the PerkinElmer Opera Phenix™ High Content Screening System. With its proprietary Synchrony™ Optics, the system enables simultaneous acquisition of the donor and the FRET image with the option to acquire two additional markers in parallel. Optimal excitation of the Cerulean donor with a 425 nm laser further increased the sensitivity in FRET measurements. Image analysis using the Harmony® High Content Analysis Software allowed an easy-to-use workflow to quantify the FRET efficiency on a pixel-by-pixel basis.
In summary, we have established a fast and robust FRET-based high content imaging assay on the Opera Phenix system that quantifies protein-protein interactions on a pixel-by-pixel basis (Z’ = 0.6).

2 Assay Principle

MCF-7 breast cancer wild type cells or MCF-7 cells stably expressing the fusion protein mCerulean3-Bcl-XL were grown in Collagen-coated CellCarrier™ Ultra 384-well plates, transfected with three different mVenus reporter constructs (Fig 1) and treated with various concentrations of ABT-737 compound. Following a 15-20 h incubation period in the presence of the compound, a live cell staining was performed using Draq5 and TMRM.

Fusion protein		FRET role	Localization	Expression
mCerulean3-BclXL	mCerulean3 – Bcl-XL	Donor	Mitochondria	Stable
mVenus-Bad	mVenus – Bad	Acceptor – **Specific Binding**	Mitochondria	Transient
mVenus-ActA	mVenus – ActA	Acceptor – **Random Collision**	Mitochondria	Transient
mVenus	mVenus	Acceptor – **Spatially Separated**	Cytoplasm	Transient

Figure 1. List of FRET reporter constructs used during this study*.

3 Ratiometric Imaging of FRET on the Opera Phenix System

Live cells were imaged on a four camera Opera Phenix system equipped with five lasers (375nm/425nm/488 nm/561nm/640nm) in confocal mode using the 40x water immersion objective. Taking advantage of all four cameras, the Draq5, TMRM, Cerulean and FRET (ex Cerulean / em Venus) images were acquired simultaneously, followed by the Venus acceptor image in sequential mode.

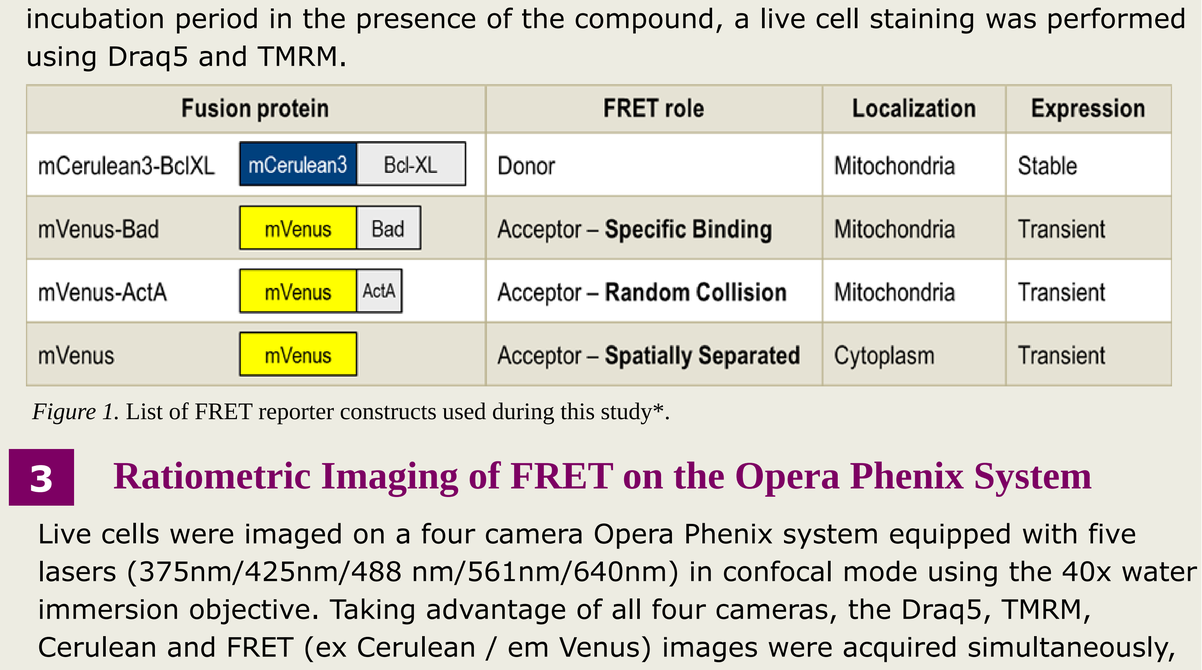

Figure 2. Ratiometric imaging of FRET on the Opera Phenix system. The NFRET pseudocolored image was calculated on a pixel-by pixel basis using the *Calculate Image* building block of the Harmony software using the formula as described by Xia and Lui, 2001. The calculated NFRET value is highest in cells transfected with mVenus-Bad (specific binding) and much lower in cells expressing mVenus-ActA (random collision) or mVenus (spatially separated). Note the different localizations of Bad and ActA (mitochondria) and Venus (cytoplasm).

4 Image Analysis Strategy using Harmony High Content Analysis Software

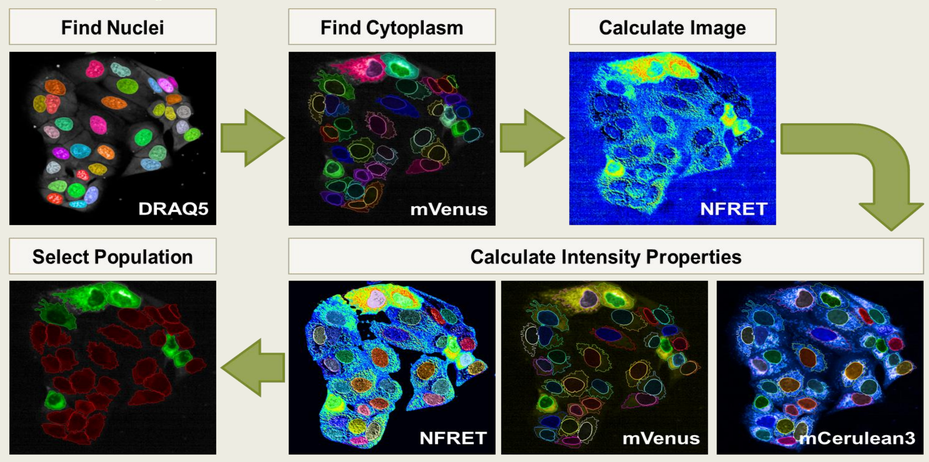

Figure 4. Image Analysis Strategy for a FRET-based protein-protein interaction assay using the Harmony software. FRET is quantified by calculating a ratiometric image using the *Calculate Image* building block with the NFRET formula as formula input. Subsequently, the mean Intensity in the cytoplasm region of the NFRET channel is calculated.

5 Comparison of the Three Different FRET Acceptors

Venus-Bad shows the highest NFRET value, as Bad is a specific interaction partner for BclXL. Venus-ActA and Venus alone are not binding specifically to BclXL and serve as negative controls. ActA is localized in the same compartment as BclXL and represents random collisions between the interaction partners. Venus localizes to the cytoplasm and is therefore a localization unmatched control.

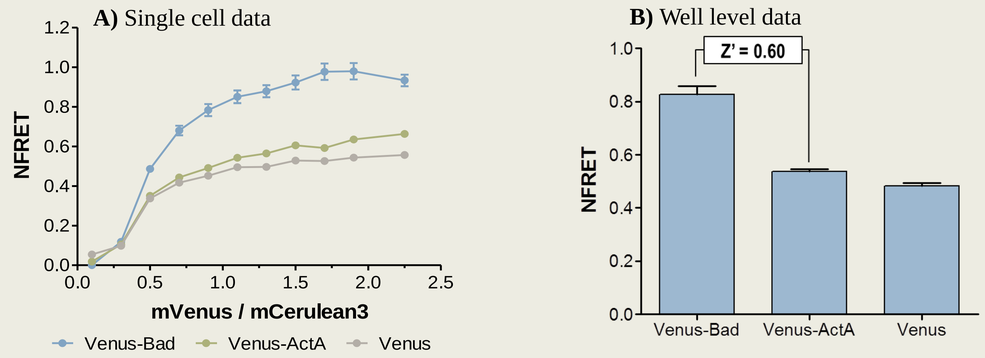

Figure 5. Binding of various Venus tagged proteins to mCerulean3-BclXL. A) The NFRET value, a measure for the interaction of FRET donor and acceptor was plotted against the ratio of mVenus to mCerulean3 intensity as measured in the cytoplasm of the cell. The more acceptor (mVenus) present in the cell, the higher the NFRET value until the donor (mCerulean) is saturated with acceptor (mVenus) (N > 40 cells).
B) The Z’ value (Z’ = 0.6), calculated using Venus-Bad as positive- and Venus-ActA as negative control suggests a robust assay (N = 3 wells).

6 ABT-737 Disrupts the BclXL-Bad Interaction

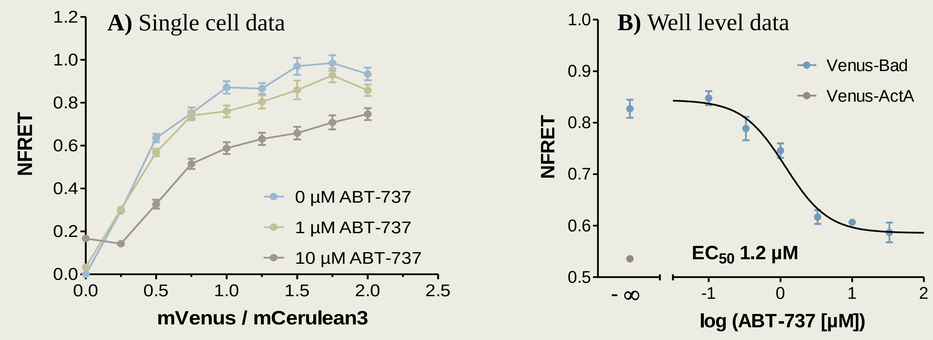

Figure 6. ABT-737 inhibits the interaction between Venus-Bad and mCerulean3-BclXL. A) Cells expressing Cerulean-Bcl-XL and Venus-Bad were treated with different concentrations of ABT-737 and the NFRET value was plotted against the mVenus/mCerulean ratio. Note the different curve characteristics for the different ABT-737 concentrations (N > 40 cells). B) ABT-737 inhibits Bad binding to BclXL in a dose dependent manner (EC50 = 1.2 µM). The localization matched Venus-ActA control represents the lower end of the curve, as the complex of ABT-737 bound to mCerulean-BclXL can still collide randomly with Venus-Bad (N = 3 wells).

7 Summary

Here, we have established a fast and robust high content FRET-based assay to study protein-protein interactions on the Opera Phenix system. The Opera Phenix equipped with five lasers (375nm/425nm/488nm/561nm/640nm) and four cameras is ideally suited for high speed acquisition of CFP/YFP FRET-based assays. The 425nm laser effectively excites the CFP donor, leading to bright FRET signals and maximized signal windows. With its proprietary Synchrony™ Optics, the system enables simultaneous confocal acquisition of the donor and the FRET image with the option to acquire two additional markers in parallel.
The image analysis is based on the easy-to-use building blocks of the Harmony software. Using the Calculate Image feature, a ratiometric image can be calculated using any FRET formula described in the literature. A ratiometric image generated this way represents the FRET efficiency on a per pixel basis, allowing analysis of the exact distribution of the protein complex of interest within the cell.

Xia, Z. and Liu, Y. (2001): Reliable and Global Measurement of Fluorescence Resonance Energy Transfer Using Fluorescence Microscopes. Biophys Journal, 81, 2395–2402.

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