

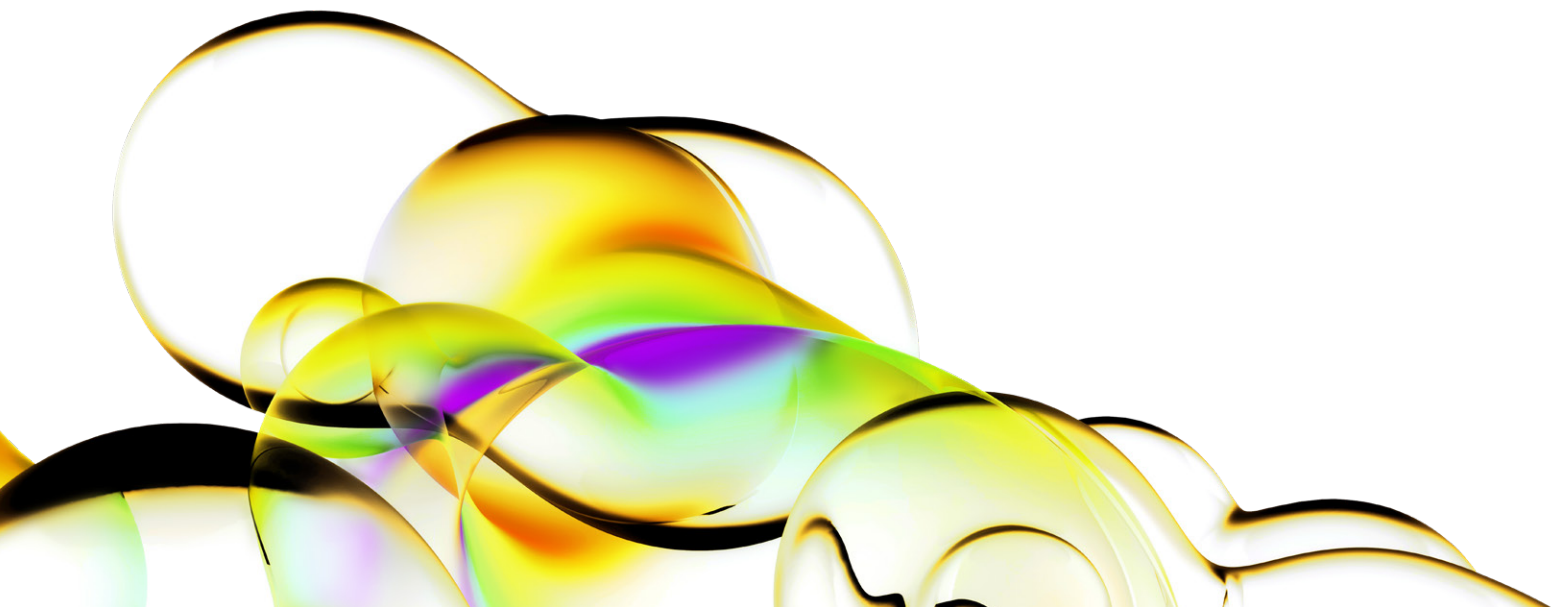
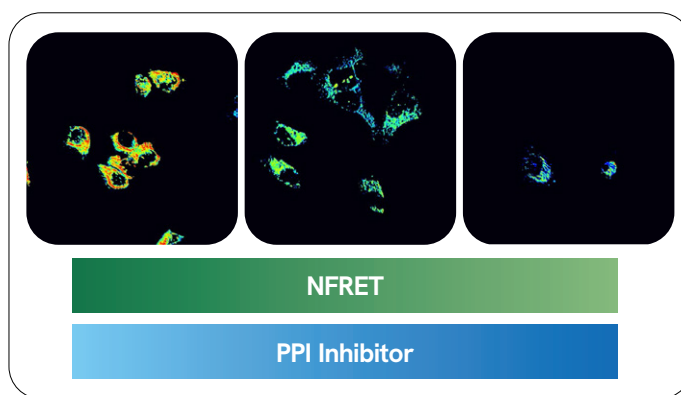
Measuring FRET using the Opera Phenix high-content screening system: A high throughput assay to study protein-protein interactions.

Key features

- Fast acquisition of FRET-based assays on the Opera Phenix® system
- Ratiometric quantification of the FRET efficiency on a pixel-by-pixel basis
- FRET analysis without expert knowledge

Introduction

Fundamental processes in living cells, such as apoptosis and signal transduction are controlled by proteins, often acting in concert with other protein partners through protein-protein interactions (PPIs). Inappropriate protein-protein recognition can fundamentally contribute to many diseases, including cancer. Therefore, inhibiting protein-protein interactions represents an emerging area in drug design. Conventional drug design has mainly focused on the inhibition of a single protein, usually an enzyme or receptor with a clearly defined ligand-binding site with which a small-molecule drug can be designed to interact. Designing a molecule that is fit to bind to a specific protein-protein interface, however, is challenging as the surface of the PPI interface is usually quite large and noncontiguous. Furthermore, a limited number of suitable high throughput screening technologies is available to characterize PPI's especially in physiologically-relevant cell models like living cells, which contributes to the notion of PPIs as difficult drug targets.



Here, we present a FRET-based high-content screening assay to study the interaction of the anti-apoptotic protein Bcl-XL with the pro-apoptotic protein Bad. Release of BH3 proteins like Bad from anti-apoptotic proteins kills some cancer cells and sensitizes others to chemotherapy (Aranovich et al., 2012). Thus disrupting the Bcl-XL or Bcl-2 interaction with pro-apoptotic BH3 proteins is of major interest to pharmaceutical companies.

By taking advantage of simultaneous confocal imaging on the four cameras of the Opera Phenix™ high-content screening system, we established a fast and robust FRET-based assay to measure PPIs. Image analysis using Harmony® high-content imaging and analysis software allowed for automated ratiometric quantification of FRET efficiency pixel-by-pixel.

FRET background

Fluorescence resonance energy transfer (FRET) is the process of energy transfer from a fluorescent donor molecule (D) to a fluorescent acceptor (A) without the involvement of a photon [Förster, 1948]. The efficiency of the energy transfer is highly dependent on the distance of donor and acceptor molecules, therefore FRET can be used to study inter- or intramolecular interactions in living cells (Fig 1). In addition to close proximity

of donor and acceptor (usually less than 10 nm), FRET requires a significant overlap of the donor emission spectrum with the acceptor absorption spectrum. Different techniques are available to measure FRET, however, the most common technique is a two- or three-channel ratiometric approach also called sensitized emission [Sun et al., 2012]. In this approach, a sample is excited with the donor wavelength while the FRET signal is detected in the acceptor channel (ex D/ em A).

As the spectra are typically close together, the FRET signal contains spectral crosstalk – donor crosstalk resulting from donor emission detected in the FRET channel and acceptor crosstalk caused by direct excitation of acceptor by the excitation source. Thus, crosstalk correction techniques may be required.

When intra-molecular FRET is measured using FRET-based biosensors, e.g. the Ca²⁺ sensor Cameleon, donor and acceptor are present at a fixed stoichiometry of 1:1 (Fig 1). In such a case, it is sufficient to perform a two-channel ratiometric experiment (ex D / em A and ex D / em D) and calculate the relative FRET efficiency as I_{FRET}/I_{Donor} . As the crosstalk signal is, in this case, proportional to the FRET signal, crosstalk correction is not required for FRET-based biosensors [Sun et al., 2012].


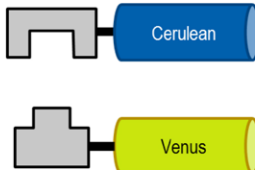
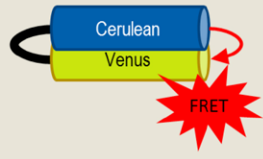
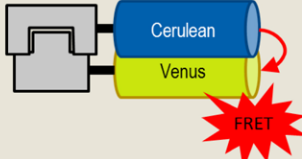
Intra-molecular FRET (FRET sensors)	Inter-molecular FRET (Protein-Protein interaction)
	
	
Fixed stoichiometry (1:1)	Varying stoichiometry
Two-channel ratiometric approach (exD / emA, exD / emD)	Three-channel ratiometric approach (exD / emA, exD / emD, exA / emA)
Crosstalk correction not required	Crosstalk correction is required
$E_{FRET} = \frac{I_{FRET}}{I_D}$ or $E_{FRET} = \frac{I_{FRET} - I_{FRET\ background}}{I_D - I_{D\ background}}$	$N_{FRET} = \frac{I_{FRET} - I_A \times \alpha - I_D \times \beta}{\sqrt{I_A \times I_D}}$ or other

Figure 1: Intra- and intermolecular FRET applications require different ratiometric imaging approaches and crosstalk corrections.

However, when inter-molecular FRET should be measured as in protein-protein interaction assays, crosstalk correction becomes important as a 1:1 stoichiometry is usually not given and the crosstalk signal is not strictly proportional to the FRET signal.

To quantify the FRET efficiency in PPI assays, numerous formulas have been developed that include crosstalk correction [Gordon et al., 1998; Xia and Liu, 2001]. We found that the N_{FRET} value as described by Xia and Lui [2001] worked best for our assay, as this method normalizes heterogeneous fluorescent protein expression levels.

$$N_{\text{FRET}} = \frac{I_{\text{FRET}} - I_A \times a - I_D \times \beta}{\sqrt{I_A \times I_D}}$$

I_{FRET} = Intensity in FRET channel

I_A = Intensity in Acceptor channel

I_D = Intensity in Donor channel

a = Cross-excitation coefficient describing percentage of acceptor crosstalk in FRET image

β = Cross-emission coefficient describing percentage of donor crosstalk in FRET image

To determine the amount of crosstalk signal, acceptor-only and donor-only control samples are necessary. These need to be measured the same way as the actual FRET samples to be able to determine the cross-excitation coefficient α and the cross-emission coefficient β (more details below in experimental protocol). On the same instrument, the crosstalk coefficients can be re-used as long as the optical setup remains unchanged.

Application

MCF-7 breast cancer wild type cells or MCF-7 cells stably expressing the fusion protein mCerulean3-Bcl-XL were seeded into Collagen-coated 384-well PhenoPlate microplates and incubated overnight (37 °C, 5% CO₂) (Aranovich et al., 2012). The next morning cells were transfected with either a MOCK plasmid or plasmids encoding mVenus-Bad, mVenus-ActA or mVenus (20 ng/well) using Lipofectamine 2000 at a 1:4 DNA:Lipofectamine ratio (please refer also to Table 1). After 6 h of incubation, the DNA-Lipofectamine complexes were removed and replaced with fresh media or with media containing 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™ (10 µM) to label the nucleus and cytoplasm and TMRM (0.5 µM) to label mitochondria. Subsequently, cells were washed twice with PBS and finally PBS was replaced with phenol red-free medium for imaging.

Live cells were imaged on a four camera Opera Phenix system equipped with five lasers (375 nm/425 nm/488 nm/561 nm/640 nm) 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.

Table 1: List of FRET reporter constructs used during this study.

Fusion protein			FRET role	Localization	Expression
mCerulean3-Bcl-XL	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

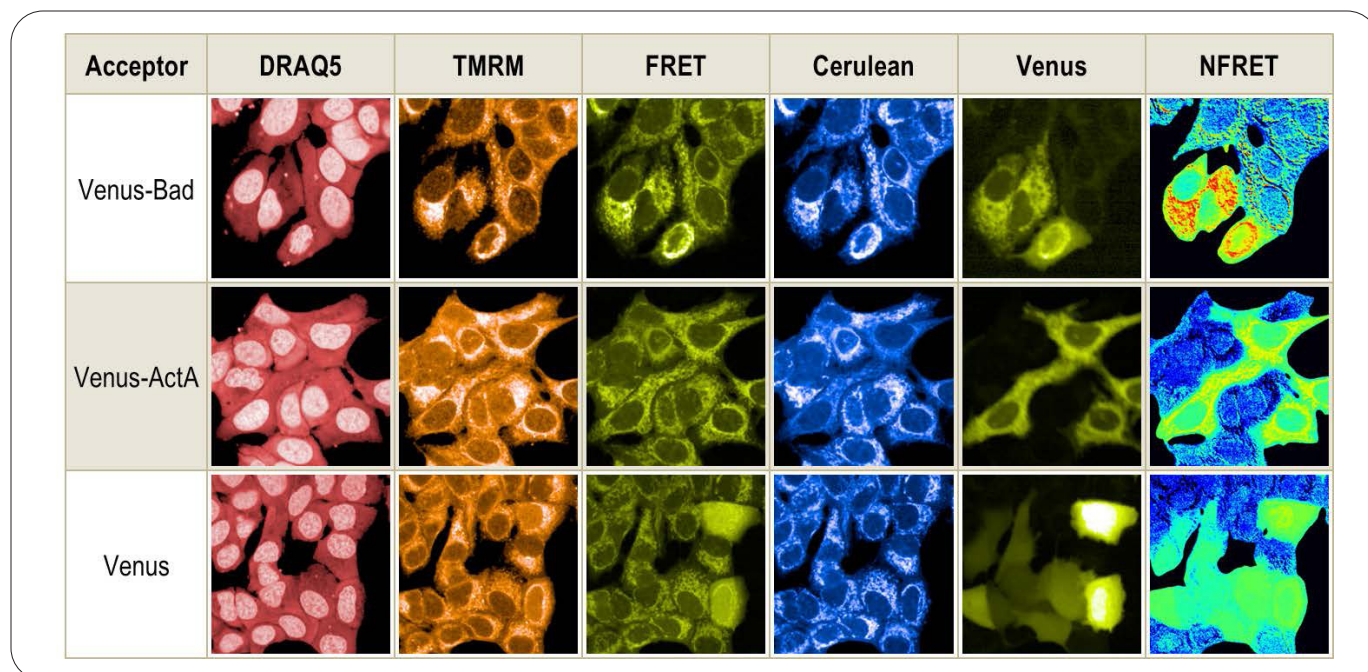


Figure 2: Ratiometric imaging of FRET on the Opera Phenix system. MCF-7 cells stably expressing mCerulean3-Bcl-XL were transiently transfected with either mVenus-Bad, mVenus-ActA or mVenus and labeled with Draq5 and TMRM. Measurement was performed in confocal mode using the 40x water immersion objective. The NFRET pseudocolored image (black-cold-hot) was calculated on a pixel-by pixel basis using the Calculate Image building block of the Harmony software. 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). Please note the different localizations of Bad and ActA (mitochondria) and Venus (cytoplasm).

As a first part of the image analysis, the crosstalk coefficients α and β were determined on the control wells. The cross-excitation coefficient α was determined by analyzing wells containing wild type cells transiently transfected with mVenus-Bad (acceptor only) while the cross-emission coefficient β was determined by analyzing cells stably expressing mCerulean3-Bcl-XL (donor only).

As a first step in the Harmony image analysis sequence, nuclei were detected on the DRAQ5 channel image using the *Find Nuclei* building block. Subsequently, the cytoplasm was detected on the Venus channel with *Find Cytoplasm*

and the mean Cerulean, Venus and FRET channel intensities were determined in the cytoplasm region using *Calculate Intensity*. To finally obtain the crosstalk coefficients, single cell results were exported and opened in TIBCO Spotfire® Platform. Crosstalk coefficients were determined by plotting the mean FRET intensity in the cytoplasm versus the mean Venus (for α) or Cerulean (for β) intensity in the cytoplasm. Subsequently, a straight line fit was applied and the slope of that straight line fit determined, as that equals the crosstalk coefficients (here $\alpha = 0.08$ and $\beta = 1.49$). The cross-emission (β) is usually much larger than the cross-excitation (α).

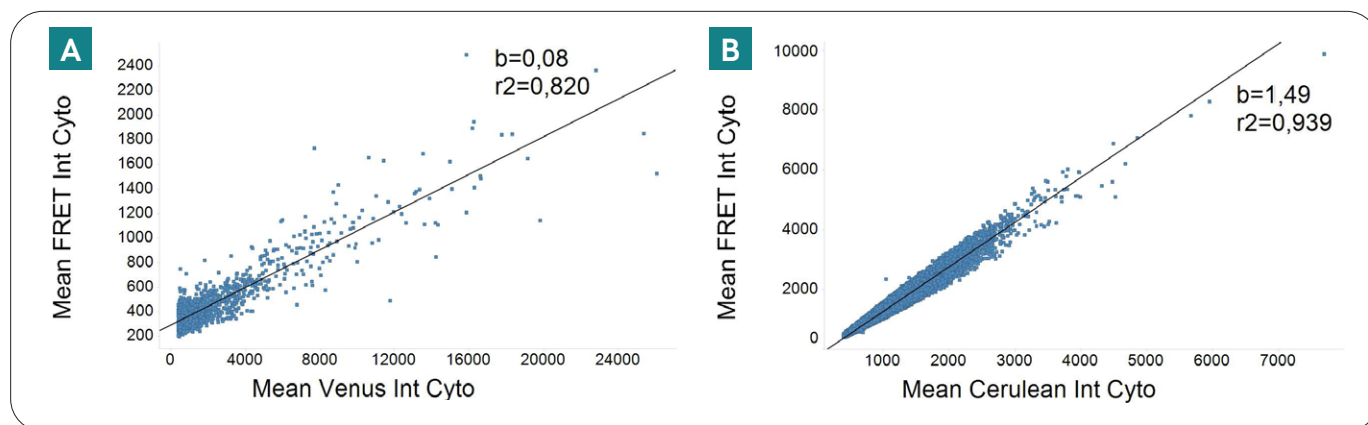


Figure 3: Determination of the crosstalk coefficients α and β using TIBCO Spotfire® Platform. A) The cross-excitation coefficient α was determined using acceptor-only expressing cells, by plotting the mean FRET intensity in the cytoplasm versus the mean Venus intensity in the cytoplasm. The cross-excitation coefficient α describes the percentage of acceptor crosstalk in the FRET image, or in other words how much of the Venus is excited by the 425 laser and is therefore wrongly measured as FRET. B) The cross-emission coefficient β was determined using donor-only expressing cells, by plotting the mean FRET intensity in the cytoplasm versus the mean Cerulean intensity in the cytoplasm. The cross-emission coefficient β describes the percentage of donor crosstalk in the FRET image, or in other words how much of the Cerulean emission is collected in the Venus channel and is therefore wrongly measured as FRET. Subsequently, straight line fits were applied to both plots and the slope determined, as that equals the crosstalk coefficients (here $\alpha = 0.08$ and $\beta = 1.49$). Usually β is much larger than α .

To study the interaction of mCerulean3-Bcl-XL with mVenus-Bad, mVenus-ActA or mVenus in the presence or absence of ABT-737 the images were segmented as described above (please refer also to Fig 4). To determine the NFRET value, a new ratiometric image was calculated by using the *Calculate Image* building block. The NFRET formula [Xia & Liu, 2001] was used as the formula input with the previously calculated crosstalk coefficients inserted. The resulting image represents the NFRET value on a pixel-by-pixel basis and also allows the quantification of protein-protein interactions in relation to their localization

in the cell. Next, three subsequent *Calculate Intensity* building blocks were used to determine the mean and maximum intensity of the cytoplasm region in the Cerulean and Venus channels and in the calculated NFRET channel. All non-expressing cells and cells with saturated pixels were excluded from the analysis using the *Select Population* building block (mean Cerulean Intensity >400, max Cerulean Intensity < 63000, mean Venus Intensity >400, max Venus Intensity < 63000, max FRET Intensity <63000). Finally, readouts were generated and single cell results stored with the *Define Results* building block.

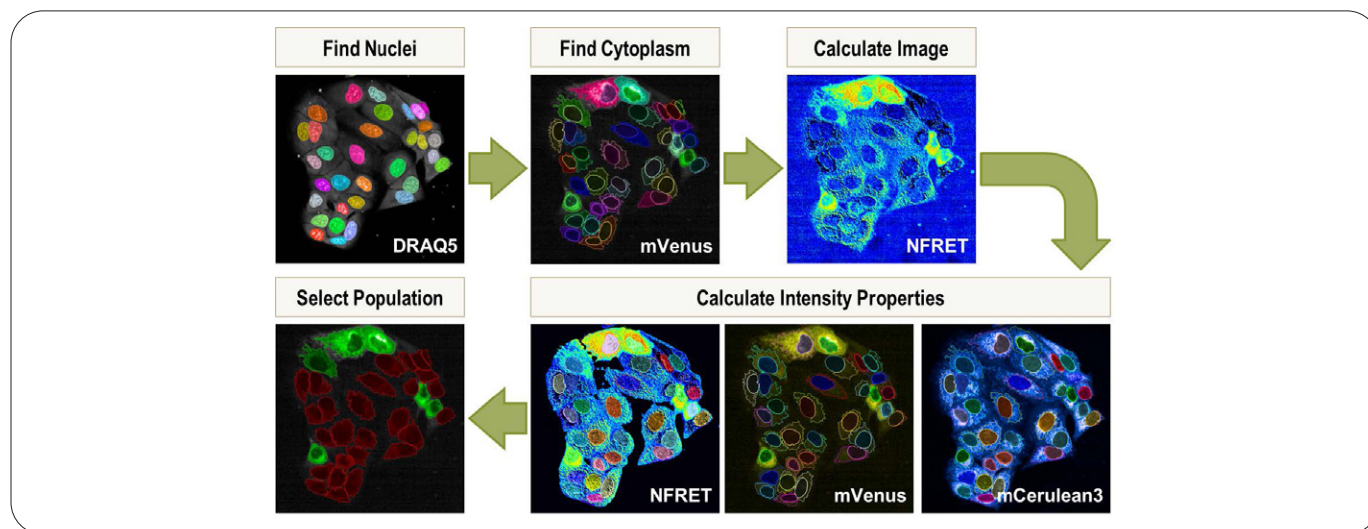


Figure 4: Image analysis strategy for a FRET-based protein-protein interaction assay. FRET is quantified by calculating a ratiometric image using the *Calculate Image* building block with the NFRET formula as formula input. Subsequently, the mean intensity in the cytoplasm region of the NFRET channel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

Results

To compare the FRET efficiency of the different FRET acceptors, the mean NFRET signal per cell was plotted versus the mVenus/mCerulean3 intensity ratio (Fig 5). The curve increases for all three acceptor fusion proteins, suggesting a correlation of the acceptor concentration with the NFRET value. The more acceptor (Venus) present in the cell, the higher the NFRET value, until the donor (Cerulean) is saturated with acceptor. As expected and previously described, the Venus-Bad curve shows the highest NFRET value, as Bad specifically binds to Bcl-XL [Aranovich et al., 2012]. Venus-ActA and Venus alone show much lower NFRET values. Venus-ActA serves as random collision control, as it localizes to the same compartment (mitochondria) as mCerulean-Bcl-XL. Venus localizes to the cytoplasm and is therefore a localization unmatched control. This plot type, sometimes also called binding curve, can be helpful during assay development to fully understand the interaction of the different partners and to determine the best assay window. Below an mVenus/mCerulean3 intensity ratio of 0.5, the assay window is quite small so further calculations were restricted to cells with a ratio of 0.5 - 2.5.

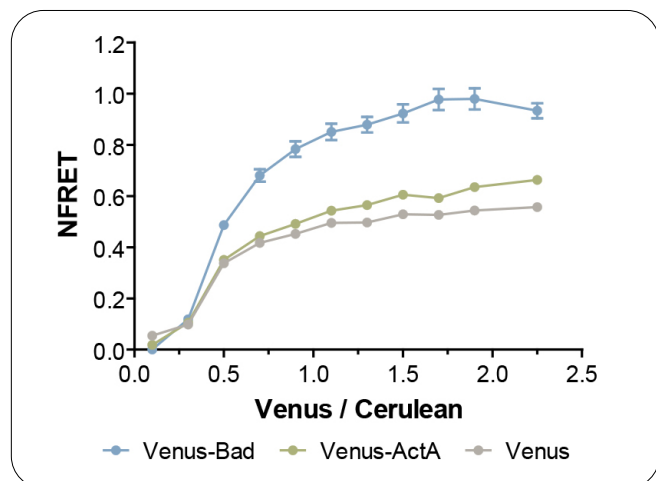


Figure 5: Binding of various Venus tagged proteins to mCerulean3-Bcl-XL measured in a ratiometric FRET imaging approach. The NFRET value, a measure for the interaction of FRET donor and acceptor, was plotted against the ratio of Venus to Cerulean intensity as measured in the cytoplasm of the cell. The more acceptor (Venus) present in the cell, the higher the NFRET value until the donor (Cerulean) is saturated with acceptor (Venus). This plot type is useful during assay development, as it can be used to determine the best assay window. Further calculations were restricted to cells with a Venus/Cerulean intensity ratio between 0.5 and 2.5 (each data point corresponds to >40 cells, the points with a ratio above 1.0 correspond to rather 1000 cells).

When averaged over all cells per well, Venus-Bad again shows the highest NFRET value (NFRET = 0.83), while the controls Venus-ActA (NFRET = 0.54) and Venus alone (NFRET = 0.48) have much lower NFRET values (Fig 6). Overall, the assay performance is very good ($Z' = 0.6$).

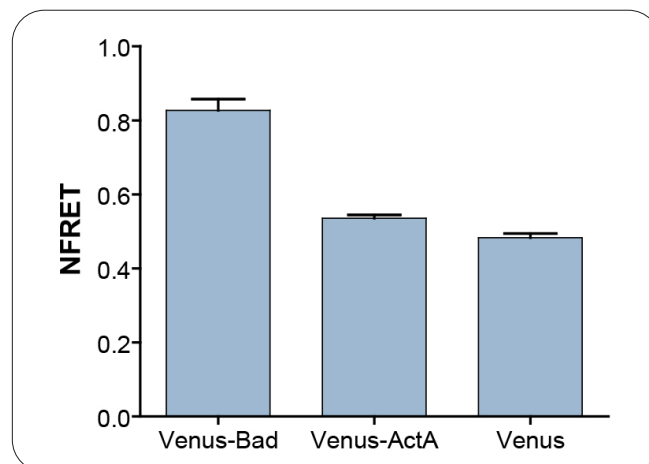


Figure 6: Quantification of protein interactions between Venus-Bad, Venus-ActA and Venus with mCerulean3-Bcl-XL. Bad shows the highest NFRET value, as it is a specific interaction partner for Bcl-XL. ActA and Venus alone are not binding specifically to Bcl-XL and serve as negative controls. ActA is localized in the same compartment as Bcl-XL and represents random collisions between the interaction partners. Venus alone is localized in the cytoplasm and therefore in a different compartment than Bcl-XL. 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).

To determine if the disruption of the Bcl-XL-Bad interaction can be measured, the mCerulean-Bcl-XL cells transiently expressing Venus-Bad or the control Venus-ActA were incubated with various concentrations of the small-molecule inhibitor ABT-737. ABT-737 is a mimetic of the Bad protein interacting with Bcl-XL and is known to enhance the effects of death signals and to display synergistic cytotoxicity with chemotherapeutics and radiation [Oltersdorf et al., 2005].

Using single cell results, the mean NFRET signal per cell was again plotted versus the mean Venus/Cerulean intensity ratio per cell. The resulting binding curves show a significant difference between untreated cells and cells treated with either 1 μ M or 10 μ M ABT-737 (Fig 7 A), suggesting ABT-737 is indeed disrupting the Bcl-XL-Bad interaction. By averaging all cells per well with a Venus/Cerulean intensity ratio 0.5 - 2.5, a dose-dependent inhibition of Bad binding to Bcl-XL could be measured (Fig 7 B).

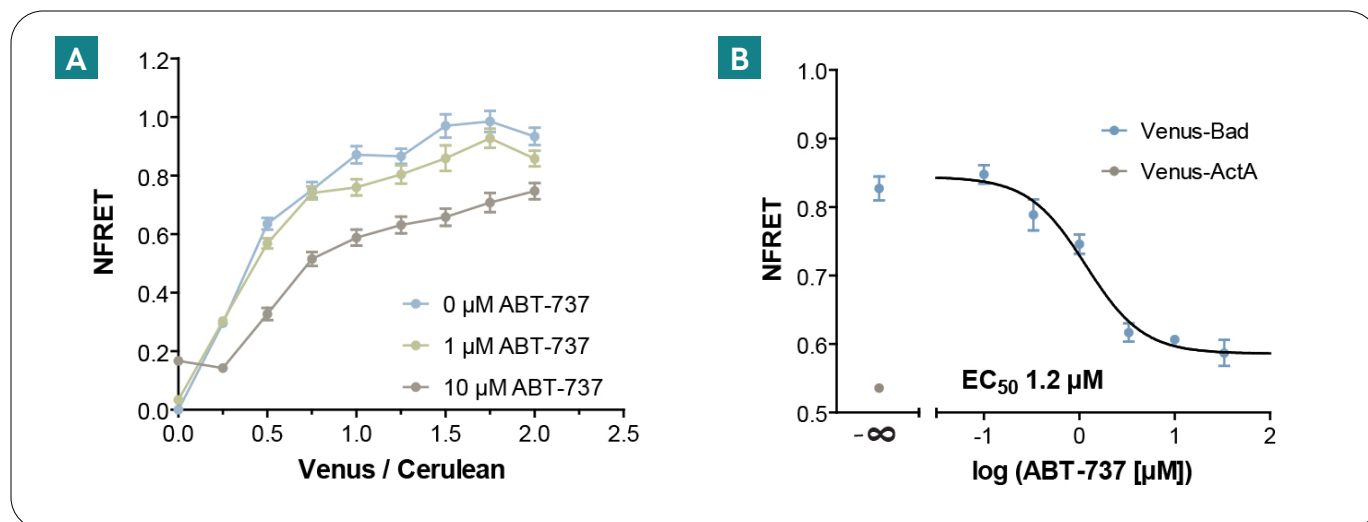


Figure 7: ABT-737 inhibits the interaction between Venus-Bad and mCerulean3-Bcl-XL. A) Cells expressing Cerulean-Bcl-XL and Venus-Bad were treated with different concentrations of ABT-737 and the NFRET value was plotted against the Venus/Cerulean ratio. Note the different curve characteristics for the different ABT-737 concentrations (N > 40 cells). B) ABT-737 inhibits Bad binding to Bcl-XL 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 Cerulean-Bcl-XL can still collide randomly with Venus-Bad (N = 3 wells).

Conclusions

In this study, 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 system equipped with five lasers (375 nm/425 nm/488 nm/561 nm/640 nm) and four cameras is ideally suited for high speed acquisition of CFP/YFP (or Cerulean/Venus) FRET-based assays. The 425 nm laser effectively excites the CFP donor, leading to bright FRET signals and potentially maximized signal windows when compared to the standard laser configuration (405 nm laser). With its proprietary Synchrony™ Optics, the Opera Phenix system enables simultaneous confocal acquisition of the donor and the FRET image with the option to acquire two additional markers in parallel and is therefore perfectly suited for ratiometric imaging of FRET-based biosensors. FRET-based biosensors are typically analyzed with a two-color ratiometric approach based on the donor and FRET channel intensities. However, protein-protein interaction assays are usually analyzed by a three-channel ratiometric approach, requiring an additional acceptor image which has to be acquired in sequential mode. Often the acceptor crosstalk, the proportion of acceptor directly excited by the donor wavelength is

negligible. When using stable cell lines expressing both donor and acceptor at constant levels, acquisition of the acceptor image can potentially be omitted. On the Opera Phenix system, this would result in a one exposure, four channel experiment, allowing to further optimize speed for screening runs.

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 gives the FRET efficiency on a per pixel basis, representing the exact distribution of the protein complex of interest within the cell. Subsequently, the encoded FRET efficiency can be quantified within any region of interest of the cell by simply quantifying the intensity of the ratiometric image. Further readouts could be calculated using the mitochondrial marker TMRM, to understand potential toxic mechanisms of the PPI disruption or potential protein redistribution towards the mitochondria. All in all, FRET-based ratiometric imaging thus becomes easy to work with, even in high throughput screening applications.

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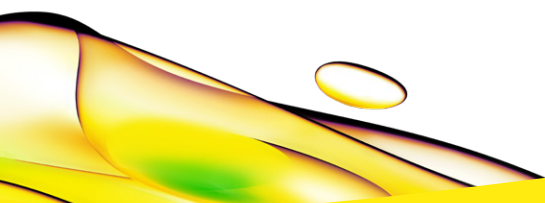
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