TechNote #TNPJ100.10

PROzyme®

PB-FRET™ *vs.* TR-FRET

Phycobiliprotein-FRET (PB-FRET) can achieve signal-to-noise ratios significantly higher than those achieved with time-resolved FRET (TR-FRET).

INTRODUCTION

In FRET assays, interaction between biomolecules is measured indirectly by conjugating one of a pair of carefully selected fluorescent dyes to each of the molecules of interest. When these fluorescent dyes are held in close proximity due to binding of the biomolecules, a unique fluorescence signal is developed that specifically confirms the proximity and thus the binding reaction (Figure 1).

To achieve resonance energy transfer, the first fluorescent molecule (the "donor") must absorb light and transfer it through the resonance of excited electrons to the second fluorescent molecule (the "acceptor"). For FRET to take place, matching of vibrational levels between electrons of the donor and the acceptor, as indicated by overlap between their emission and excitation spectra, respectively, must take place. This transfer is nonradiative in nature: that is, it does not involve the emission and reabsorption of a photon, but rather takes place through resonance-based transfer of excitation energy.

The degree of proximity and the geometry of the molecules determine the extent to which energy transfer takes place. If energy transfer were complete, fluorescence of the donor molecule would be completely extinguished, and all fluorescence would appear at the emission wavelength of the acceptor. In practice, complete transfer is never achieved, although tandem conjugates of fluors (two fluorescent molecules conjugated to one another) can achieve greater than 90% elimination of donor fluorescence.

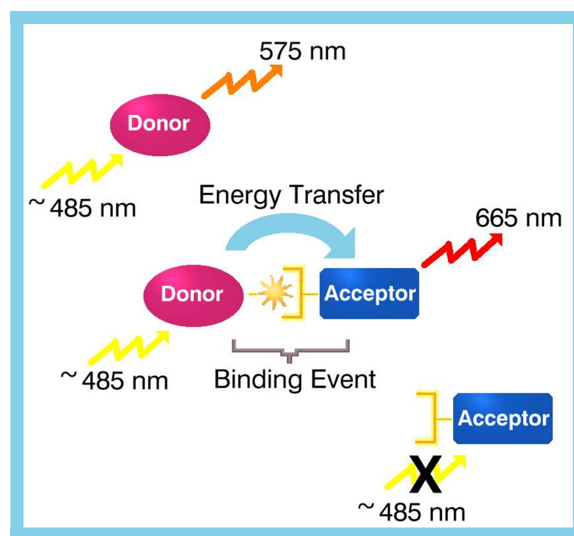

Figure 1 - Measuring a binding event with PB-FRET

In FRET proximity assays, it is the appearance of acceptor fluorescence in response to excitation of the donor molecule that indicates that the molecular interaction of interest has taken place. However, donor quench may provide valuable information and should not be ignored.

Figure 2 shows the fluorescence excitation and emission curves of two phycobiliproteins that can be useful in PB-FRET assays; R-Phycoerythrin (RPE) as the donor, and Allophycocyanin (APC) as the acceptor. It shows the characteristics of donor-acceptor pairs that must be considered when selecting dyes for this use:

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Emission spectrum overlap - Since complete quenching of donor fluorescence cannot be expected, the emission wavelength of the acceptor should be located at a wavelength where fluorescence of the donor is as low as possible, since direct fluorescence by the donor can create a significant background signal, the magnitude of which is sensitive to the extent of energy transfer (region II, Figure 2).

Excitation spectrum overlap - The acceptor molecule should be minimally excited by the wavelength employed to excite the donor. In the case of RPE, a secondary excitation peak provides a particularly suitable region for excitation in the proximity assay (region I, Figure 2).

See TechNote TNPJ100.23 *PB-FRET™: Illumination & Detection Windows For Filter-based Instruments* for further discussion of emission and detection wavelengths.

TR-FRET Assays

Assays based on the use of europium (Eu) or other lanthanides as the donor molecule benefit from one particular characteristic of lanthanide fluorescence, a very long fluorescence lifetime. Where phycobiliproteins and many other dyes release photons within nanoseconds of photoexcitation, lanthanide fluors release photons over milliseconds. Since electrons remain in the excited state until photons are released or energy transfer takes place, FRET is also prolonged over the fluorescence decay period of the lanthanide. Thus, by introducing delay between excitation and measurement of emission, acceptor fluorescence due to FRET can be distinguished from acceptor fluorescence due to off-peak excitation and from background sample autofluorescence. Assays that take advantage of the extended fluorescence lifetimes of lanthanides are commonly called time-resolved FRET (TR-FRET) assays.

Unfortunately, lanthanide-based fluors have several drawbacks as FRET donors. Unlike phycobiliproteins, which have been evolutionarily optimized for the absorption of light, lanthanides, even when enhanced by incorporation in cryptates or chelates, are poor antenna molecules. Thus, their ability to absorb light to feed the FRET process is

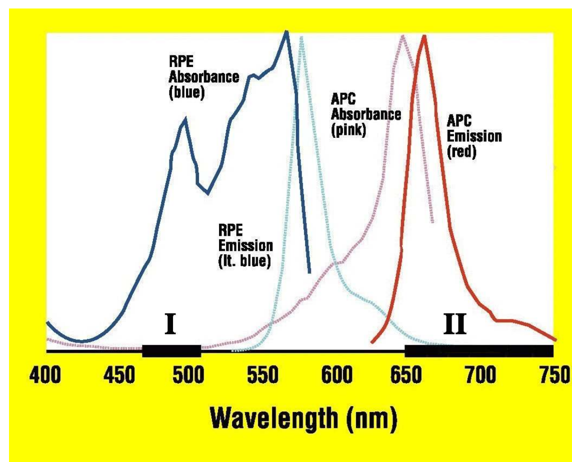

Figure 2 - Absorbance and emission spectra of RPE and APC

extremely limited. In addition, a long fluorescence decay period means that photons are released slowly; photons released before and after the time window for counting are not included in the measurement, further reducing the effective fluorescence intensity. The lower intensity translates into either a weaker signal, or an increased reagent requirement.

A phycobiliprotein, allophycocyanin (APC, sometimes called XL665), is routinely paired with fluorescent europium chelates or cryptates. APC acts as the acceptor in these assays, where its multiple fluorophores enhance its ability to successfully receive energy transfer from the donor europium. In addition, the emission peak of APC falls in a trough between the multiple emission peaks of europium (Figure 3).

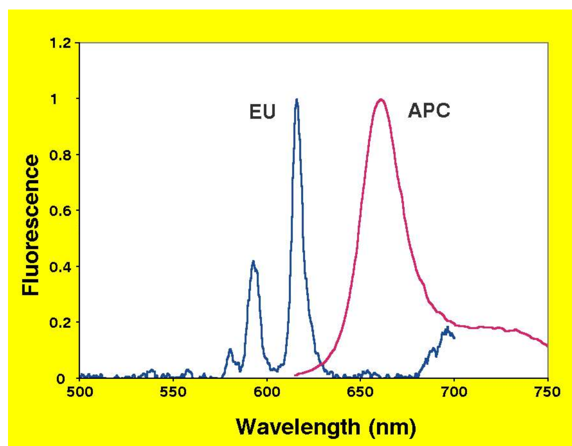

Figure 3 - Emission spectra of Eu and APC

PB-FRET *vs.* TR-FRET: Performance in a Phosphorylated Peptide Assay

Methods - Methods employed for the detection of tyrosine kinase phosphorylated peptide are described in detail in TechNote TNPJ100.11 *Detection of Tyrosine Kinase Phosphorylated Peptide With FRET.* We report here a comparison of PB-FRET and TR-FRET results in this assay. Standard TR-FRET protocols as recommended by the manufacturer of the fluorescent plate reader and lanthanide-chelate reagents were employed. For PB-FRET, we used optimized methods recommended by ProZyme, with the exception that PB-FRET counting time was restricted to 0.1 sec in order to provide comparable throughput for the two methods. Method details are shown in Table 1. (Various parameters that can be manipulated to increase PB-FRET assay sensitivity are discussed in more detail in TechNote TNPJ100.15 *Optimization of S/N Ratios in PB-FRET.*

Results - The levels of and variability in raw counts obtained in the two assays are illustrated in Figure 4; average counts for all measurements and blanks are summarized in Table 2. The most salient feature of these results is the significantly higher count level obtained in the PB-FRET assay. Since higher counts have lower relative standard deviations (TechNote TNPJ100.02 *Precision of FRET Assays: S/N vs. S/B Ratios in FRET Assays*), variability (noise) in calculated FRET values is significantly higher in TR-FRET than in PB-FRET, as reflected in the signal-to-noise (S/N) and Z′ results in Table 2 (see TechNote TNPJ100.04 *FRET Calculations*).

Table 1 - Assay Conditions

	PB-FRET	TR-FRET
Donor	SA-RPE 2.3 nM	PY20-Eu 6.0 nM
Acceptor	PY20-APC 6.0 nM	SA-APC 2.3 nM
Incubation	2 h	2 h
Excitation	485/15	340/50
Emission A	665 LP	665/15
Emission B	600/25	615/8.5
Flash interval	N/A	1000 μsec
Read duration	100 msec	100 μsec
Read delay	None	50 μsec
Iterations	1x	95 - 100x
Total time	100 msec	100 msec
Plates	White/96	Black/96

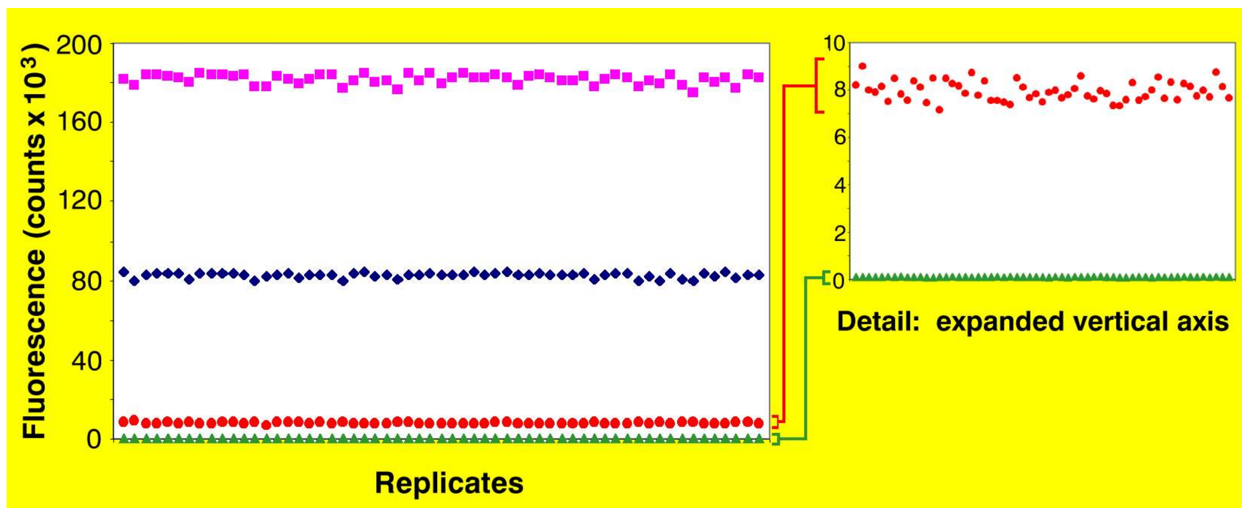

Figure 4 - Counting levels and variability in selected measurements for two FRET methods.
T_a, PB-FRET (■); C_a, PB-FRET (◆); T_a, TR-FRET (●) and C_a, TR-FRET (▲)

Table 2 - Measured and Calculated Values for PB-FRET and TR-FRET
Means of five replicate counts of twelve replicate wells for each value.

	Mean	STD	Rel STD	Mean	STD	Rel STD
Measured Values	PB-FRET			TR-FRET		
T_a - Test, Channel A	181,494	1239	0.7%	7,939	245	3.1%
C_a - Assay Control, Channel A Both Donor and Acceptor present but no FRET *i.e.* no peptide or no kinase	82,560	491	0.6%	96	9	9.7%
A_a - Acceptor Blank, Channel A All reagents except donor	45,036	340	0.8%	8	3	39.4%
D_a - Donor Blank, Channel A All reagents except acceptor	67,014	401	0.6%	96	10	10.0%
I_a - Instrument/Buffer Blank, Channel A	32,909	287	0.9%	7	3	48.9%
T_b - Test, Channel B	181,710	969	0.5%	23,589	697	3.0%
C_b - Assay Control, Channel B Both Donor and Acceptor present but no FRET *i.e.* no peptide or no kinase	304,658	1192	0.4%	27,926	382	1.3%
A_b - Acceptor Blank, Channel B All reagents except donor	72,160	519	0.7%	16	5	29.4%
D_b - Donor Blank, Channel B All reagents except acceptor	316,359	965	0.3%	28,907	382	1.3%
I_b - Instrument/Buffer Blank, Channel B	74,067	562	0.8%	14	4	28.1%
Calculated Values (see TechNote TNPJ100.04 *FRET Calculations)*						
Net FRET Counts	116,240	1423	1.2%	7,856	380	4.8%
Quench Counts	122,949	1075	0.9%	4,336	200	4.6%
Quench %	40%			16%		
Raw Channel A/Channel B	0.728	0.008	1.2%	0.333	0.011	3.2%
Signal/Noise (S/N) of Net FRET	94	14	14.5%	33	3	9.0%
Z′ of Net FRET	0.96			0.90		
Signal/Noise (S/N) of Quench	81	9	11.2%	5.8	1.1	19.7%
Z′ of Quench	0.95			0.29		
Signal/Noise (S/N) of A:B	121	35	28.6%	63	19	30%
Z′ of A:B	0.97			0.94		
Raw Signal/Background (S/B) of Net FRET	2.2	0.012	0.5%	83	4	4.5%
Raw Signal/Background (S/B) of A:B	3.7	0.22	0.6%	98	5	4.9%

DISCUSSION

The differences between PB-FRET and TR-FRET can be summarized by saying that PB-FRET gives significantly higher counts for all wells, both in test wells and in blanks. It is worth noting that these results were obtained with a Wallac Victor^2 1420 Multilabel Counter (PerkinElmer), in which the continuous lamp used for PB-FRET is much weaker than the flash lamp used for TR-FRET. Even higher PB-FRET counts would be expected in instruments with stronger continuous lamps. Selection between the two methods rests on determining whether the benefits of the higher overall signal outweigh the costs of a higher blank.

Low background readings for TR-FRET have been offered as a major advantage of the technology for two reasons:

- because low "signal-to-background", *i.e.* raw signal-to-background (S/B) has been equated with low S/N, and
- because time-delayed readings are less subject to interference from sample autofluorescence, which decays rapidly.

Thus, in assessing the usefulness of PB-FRET, these two questions have to be answered:

Does low S/B ensure low S/N? The answer here is clear: No. The data shown in Table 2 make it clear that while the various measures of FRET (Net FRET, Quench, A:B) in PB-FRET have significantly lower S/B than those for TR-FRET, S/N for each of these parameters is significantly higher. S/B is simply one of many factors that influence S/N; in the results shown here, its influence is swamped by the massive benefit derived from higher counts and associated lower variability in PB-FRET. (These concepts are discussed in more detail in TechNote TNPJ100.02).

Do low background counts make it easier to identify extraneous signals? Not necessarily. The answer here rests on a fundamental feature of FRET: that FRET acceptor fluorescence occurs in direct proportion to FRET donor quenching. Samples in which FRET occurs will display Quench and Net FRET in the expected proportion; samples in which this proportion is not maintained can be identified as suspect or eliminated outright, depending on values obtained (see TechNote TNPJ100.19 *Detecting Interference in PB-FRET* for a detailed description of FRET-Quench analysis.)

For this technique to be effective, however, reliable (low-noise) values for both Net FRET and Quench must be obtained. Table 2 shows that the S/N in the PB-FRET assay for Net FRET and Quench are 94 and 81 respectively, while the same values in the TR-FRET assay are 33 and 6. The feasibility of simultaneous Quench and FRET analysis in PB-FRET makes it possible to identify not only sample autofluorescence in either or both channels, but also excitation quenching by the sample and emission quenching by the sample in either channel. TR-FRET data, while it excludes most autofluorescence, is less suitable for comparable analysis due to noisier data, particularly in Quench values.

In addition to providing more informative data, PB-FRET offers logistic advantages as well. Since phycobiliproteins are extremely popular reagents in flow cytometry and other applications, and have been in use for years, hundreds of phycobiliprotein-conjugated antibodies are currently on the market, as well as activated pigments, conjugation kits and unconjugated phycobiliproteins, all at competitive prices. Many vendors are also prepared to perform custom conjugations. The conjugation technology is widely licensed and has no association with a particular equipment manufacturer. Conjugates with prepaid licensing are also available. In short, PB-FRET is based on a mature market where proprietary technology and licensing issues are highly manageable; specifically, there are no Technology Access Fees, or Reach-Through Royalties.

Table 3 - PB-FRET™ *vs.* TR-FRET

Property	PB-FRET	TR-FRET
Homogeneous/readily automated	Yes	Yes
Donor	Strong donor absorbance & brightness	Weak donor absorbance & brightness
Acceptor (same for both)	Good acceptor brightness	Good acceptor brightness
Detection window	Broader emission window obtainable	Selection of detection window severely constrained by multiple lanthanide emission peaks
Detection delay	No	Yes
Multiple reagent suppliers		
Donor	Yes	No
Acceptor	Yes	Yes
Signal	Strong	Weak
Background	Higher	Lower
Noise	Decreased due to strong signal, increased due to higher background	Increased due to weak signal, decreased due to low background
Signal/Noise	Excellent	Good

CONCLUSIONS

PB-FRET, by virtue of employing the most strongly fluorescent of commercially available fluorescent tags, provides a higher S/N alternative to TR-FRET. In addition, the established market in phycobiliprotein conjugates helps to ensure reliable and cost-effective reagent availability. Table 3 summarizes the comparison of the two methods.

TECHNICAL SERVICE

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<http://www.prozyme.com /technical/index.html#technotes>

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