

Monitoring receptor ligand binding in living cells

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- cAMP BRET biosensor system applied to live cells
- High sensitive LVF monochromator of the CLARIOstar[®] microplate reader enables detection of BRET emission signals without using filters

Introduction

Bioluminescence resonance energy transfer (BRET) is an established method to study protein-protein interactions. Most of the research focuses on GPCRs as these receptors play an important role in finding new drug candidates.¹

There are several BRET-based approaches that allow the monitoring of protein-protein interaction² such as:

- BRET between receptors and ligands³
- BRET between receptors and G proteins
- BRET between receptors and downstream effectors, such as adenylyl cyclase or ion channels
- BRET sensors that follow beta-arrestin recruitment
- cAMP BRET biosensors

In this application we will show how a cAMP BRET biosensor can be used to monitor ligand binding with the help of the CLARIOstar microplate reader.

Assay Principle

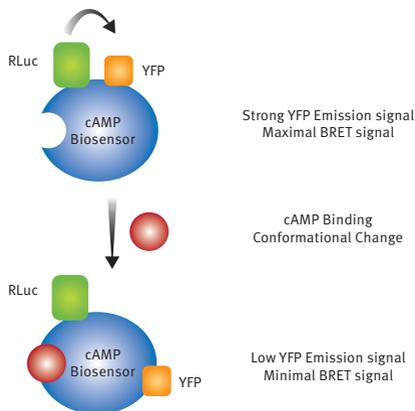


Fig. 1: Assay Principle of detection of ligand binding using BRET1 technology.

The BRET biosensor carries a cAMP binding domain, *Renilla* luciferase and YFP. The latter two are in close proximity. Once the luciferase is activated by substrate addition [Coelenterazine H] the enzyme emission excites YFP. This leads to a strong emission signal at 530 nm and therefore to a strong BRET signal [Fig. 1]. Once a ligand is binding to a GPCR, G proteins are activated and cAMP is generated. Free cAMP will find its binding site and the biosensor will undergo a conformational change leading to a greater distance between RLuc and

YFP. The result of that process is no or only weak YFP emission leading to a small BRET signal.

Once ligands are found that are able to effectuate the conformational change, a dose response can be run in order to determine EC50 values.

Materials & Methods

- White 96-well microplates from NUNC
- Coelenterazine h from Dalton Pharma Services
- CLARIOstar microplate reader from BMG LABTECH

cAMP biosensors were prepared as described in reference 2. Cells were transiently transfected with the fusion plasmids. One day before BRET experiments the cells were seeded into 96-well microplates.

CLARIOstar settings for ligand binding

Detection Method	Luminescence, plate mode, top reading
Optic setting	Well mode chromatics selected
Monochromator	Just select 475-30 and 535-30 nm, the bandwidth is adjustable up to 100 nm
Filters	Emission filters 475-30 and 535-30 nm
Kinetic settings	No. of cycles: 10 Cycle time: 90 sec Measurement time: 0.50 sec
Gain	3600 for both channels

BRET calculation

- Ratio calculation: 535 nm / 475 nm
- Average of 4 first measurement points (based on ratio) = A0
- Average of 5 last measurement points (based on ratio) = At
- Divide At/A0
- Use color gradient to see positive results immediately (an example is shown in Fig. 3)

Results & Discussion

Initial BRET luminescence scan

Two controls were prepared. One well contained the biosensor without ligand – so a high BRET signal is expected [BRET_{Max} Control]. A second well consisted of the cAMP biosensor accompanied by a high ligand concentration. It was a ligand concentration that is known to lead to an increase in cAMP levels and therefore into a conformational change. For the latter a minimal BRET signal is expected [BRET_{Min} Control]. Overlaid luminescence spectra are shown in Fig. 2.

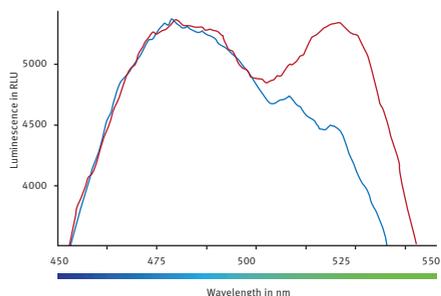


Fig. 2: CLARIOstar LVF monochromator luminescence scan from 320-600 nm with a resolution of 1 nm. The red line corresponds to the BRET_{max} Control, the blue line is the spectral result of the BRET_{min} Control. In the MARS Data Analysis software the moving average function was applied (factor of 5) to smooth the curve.

Binding of ligand to receptor

Different ligands can be tested on one microplate. A color gradient was applied in the MARS Data Analysis software to get a fast overview of wells in which the ligand was effectively binding (Fig. 3).



Fig. 3: BRET1 ratio values obtained with the CLARIOstar. A color gradient was applied using green to indicate a successful ligand binding and red to show a negative result.

Only forskolin treated samples showed a decrease in BRET ratio. Forskolin is known to directly activate adenylylate cyclase which in turn produces cAMP.

Therefore, forskolin can be used as a control substance for this assay. A dose response curve was created by adding varying amounts of forskolin in replicate wells (Fig. 4)

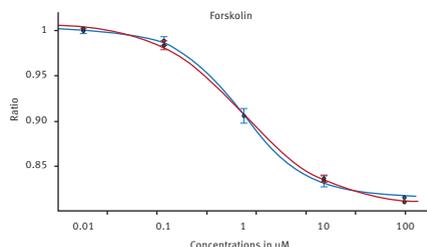


Fig. 4: Forskolin dose response curve obtained with the CLARIOstar. The blue line is based on monochromator measurements while the red line is based on filter measurements.

Although the intrinsic assay window is quite small, the CLARIOstar is sensitive enough to detect small luminescence changes by either using BRET specific emission filters or by using the LVF monochromator. The standard deviation in replicates is small enough to obtain excellent 4-parameter dose response curves with $R^2 > 0.999$.

Conclusion

The CLARIOstar microplate reader is a suitable instrument to detect protein ligand binding with the help of the cAMP BRET biosensor technology. The MARS Data Analysis software can be set up to use a color gradient to fast identify ligands that lead to a receptor conformational change.

References

- 1) Kocan and Pflieger [2011] Study of GPCR-protein interactions by BRET. *Methods Mol. Biol.* **746**: 357-371.
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- 3) Stoddart et al. [2015] Application of BRET to monitor ligand binding to GPCRs. *Nat. Methods* **12**(7): 661-663.



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