

NanoBRET™ assay for monitoring of ligand binding to GPCRs in live cells, using the CLARIOstar® and the PHERAstar® FS

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- First use of BRET for monitoring ligand binding to GPCRs in live cells enabled by NanoLuc® luciferase
- IC_{50} and K_D values were calculated from saturation and competition binding assays

Introduction

Due to the crucial role of G protein-coupled receptors (GPCRs) in mediating cellular responses to external stimuli, these receptors have been and will remain a prime focus for medical research and the pharmaceutical industry for many years to come. Their importance is especially highlighted by the statistic that 30-50% of marketed pharmaceuticals target GPCRs.

Current assay techniques for studying ligand binding to GPCRs include use of radioactivity, fluorescent ligands and FRET (fluorescence resonance energy transfer). BRET (bioluminescence resonance energy transfer)¹ requires only the addition of a suitable substrate to generate donor light emission, as well as energy that can excite fluorescently-labelled ligands in a non-radiative manner. The donor luciferase enzyme can be expressed on the N-terminus of the receptor of interest in an appropriate transfected cell line. Theoretically, BRET should be more sensitive than FRET-based assays, however the practical difficulty of finding a suitable luciferase enzyme amenable to such N-terminal fusion has hampered the development of a BRET assay for ligand binding studies.

Recently, researchers at Promega have developed a genetically engineered luciferase called NanoLuc® (Nluc) from a natural luciferase isolated from deep sea shrimp.² Nluc is of relatively small molecular weight (19kDa) and is very stable (up to 55°C). Unlike other luciferase-GPCR fusion constructs, it is readily expressed on the N-terminus of GPCRs and efficiently transported to the cell membrane.³

Assay Principle

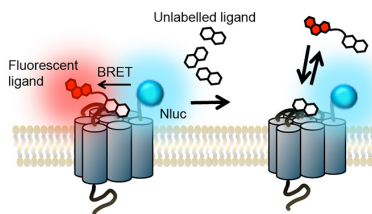


Fig. 1: The NanoBRET™ binding assay principle.

In this particular example, a GPCR is expressed in live cells with Nluc luciferase on its N-terminus.³ A ligand of this receptor is then labelled with a fluorophore that has an excitation spectrum overlapping the emission spectrum of Nluc. The ligand is introduced to the live cells

followed by addition of the Nluc substrate (furimazine). If the ligand does not bind to the receptor, only the blue emission of Nluc is detected. If the ligand binds specifically to the receptor, excitation of its fluorescent label by resonance energy transfer from the Nluc results in red fluorescence also being detected. Competitive binding of unlabelled ligand then causes a concentration-dependent decrease in this red fluorescence (Fig. 1).

Materials & Methods

Detailed materials and methods are provided in the Online Methods of Stoddart et al.³

Stable cell line generation: The HEK293 cell line expressing β_2 adrenergic receptor (β_2AR) was from Promega.

Fluorescently labelled ligands: Alprenolol-TAMRA was synthesised by Promega; propranolol-BY630 was obtained from CellAura; alprenolol was obtained from Sigma; isoprenaline, propranolol, ICI 118551 and CGP 12177 were obtained from Tocris.

Nluc and Rluc8 emission spectra: 24 h post-transfection, cells expressing either Nluc- β_2AR or Rluc8- β_2AR were incubated for 180 min at 37 °C in OptiMEM without phenol red (Gibco) in 96 well plates. The luciferase substrates furimazine (Nluc) or coelenterazine h (Rluc8), at a final concentration of 10 μ M, were then added to the wells.

BRET β_2AR -ligand binding assays: HEK293 cells transfected with Nluc- β_2AR were assessed in 96 well plates. For saturation binding experiments using alprenolol-TAMRA, serially diluted alprenolol-TAMRA in the absence or presence of 10 μ M alprenolol was added to the wells and the plate incubated for 120 min at room temperature. The furimazine substrate was then added to a final concentration of 10 μ M. BRET was measured at room temperature. For the competition binding experiments, Nluc- β_2AR stably transfected HEK293 cells were incubated with 10 nM propranolol-BY630 and the required concentration of competing ligand diluted in HEPES-buffered saline solution for 1 h at 37 °C.

Instrument settings

Nluc and Rluc8 emission spectra: The emission spectra were determined with a CLARIOstar® plate reader using the luminescence scanning option of the monochromator (20-nm bandwidth; 1-nm resolution; integration time: 500 ms; gain: 3000).

BRET β_2AR ligand binding assays:

For the saturation binding experiments using alprenolol-TAMRA, the CLARIOstar plate reader was used in endpoint mode, using emission filters 450±40 nm (80-nm bandpass) and >610 nm (longpass). The wavelengths were read sequentially for each well (well multichromatics) at room temperature (integration time: 500 ms; gain: 3000 for both channels).



The PHERAstar® FS was used for the competition binding experiments and the saturation binding experiments with propranolol-BY630; endpoint mode, NanoBRET™ module (460 nm (80-nm bandpass) and >610 nm (longpass)). Both wavelengths were measured simultaneously at room temperature (integration time, 1000 ms; 460 nm gain, 2800; >610 nm gain, 3000).

The raw BRET ratio was calculated by dividing the >610-nm emission by the 460nm emission. The term “raw BRET ratio” is used, as no background ratio was subtracted.

Results & Discussion

In initial experiments, sets of HEK293 cells expressing the β_2 AR N-terminally-labelled with either Rluc8 or Nluc were both found to give a luminescent signal on addition of their respective substrates. The Nluc signal maximum was at 460 nm, compared to 480 nm for Rluc8 (Fig. 2). However, the relative signal strength was about 70 times greater for Nluc compared to Rluc8 [see data in Stoddart et al.³].

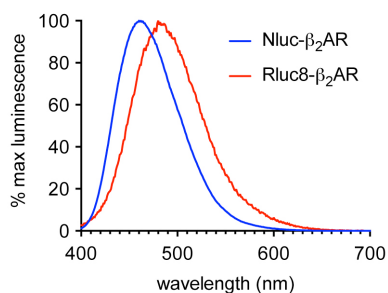


Fig. 2: Normalised emission spectra of HEK293 cells expressing β_2 AR N-terminally labelled with either Nluc (blue) or Rluc8 (red) using the CLARIOstar. Data previously published in Stoddart et al.³

Although the emission spectrum of Nluc has a maximum at 460 nm, it has appreciable intensity to ~600 nm (Fig 2). This allows the use of a range of fluorescent labels on the agonist/antagonist GPCR binding partners of interest. For HEK293 cells expressing Nluc- β_2 AR, the β_2 AR antagonist alprenolol was labelled with TAMRA [Ex 565, Em 580].

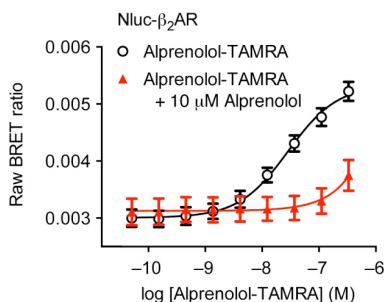


Fig. 3: Saturation binding experiment using HEK293 cells expressing Nluc- β_2 AR with increasing concentrations of alprenolol-TAMRA (black) and with increasing concentrations of alprenolol-TAMRA in the presence of a high concentration of unlabelled alprenolol (red). Data previously published in Stoddart et al.³

Specific ligand binding was observed, which could be completely prevented by competition with unlabelled alprenolol (10 μ M) (Fig. 3).

A second fluorescent ligand was then assessed, propranolol-BY630. From saturation binding experiments with this labelled antagonist binding to the receptor, an equilibrium binding constant [K_D] of 18.9 ± 4.1 nM was calculated.

Most importantly, the BRET signal that was generated from the interaction of propranolol-BY630 with the receptor could be decreased by increasing concentrations of the unlabelled β_2 AR antagonists propranolol, ICI 118551 and CGP 12177, and the agonist isoprenaline (Fig.4). From these experiments, pKi values could be calculated from the IC_{50} values of the three antagonists propranolol, ICI 118551 and CGP 12177 (8.13 ± 0.05 , 8.04 ± 0.04 and 8.32 ± 0.03 respectively).³ Furthermore, these values were similar to those published using radioligand binding experiments,⁴ highlighting the suitability of this assay for drug discovery and profiling.

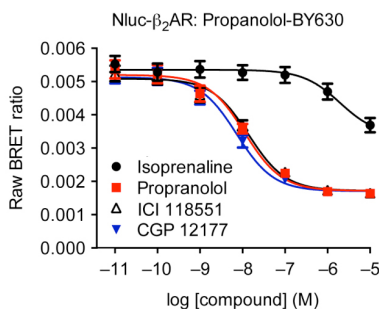


Fig. 4: Competitive binding experiments of known unlabelled β_2 AR ligands. Data previously published in Stoddart et al.³

Conclusion

The above study firmly establishes the NanoBRET™ assay as a viable alternative to existing methods for determination of ligand binding to GPCRs. The method will be useful in basic research as well as the pharmaceutical industry.

References

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