A Functional BRET Assay for 7TM Receptors Using the Mithras LB 940 Multimode Plate Reader

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Introduction

7TM (seven transmembrane segment) receptors, also known as G-protein coupled receptors, constitute the largest super-family of proteins in our organism and are targets for a large number of intercellular messengers such as hormones and transmitters. 7TM receptors are under the focus of extensive investigation as they represent both potential and established drug targets. Upon stimulation by agonists a 7TM receptor becomes activated and intracellular signalling events are initiated. The intracellular signalling is complex and involves multiple specific processes. The interaction between agonist-activated receptor and β-arrestins is an almost universal phenomenon, which has been shown to occur for the vast majority of G-protein coupled receptors (GPCRs). When Rluc (Renilla Luciferase) is fused to the C-terminus of a 7TM receptor and GFP^2 (Green Fluorescent Protein 2) is fused to the N-terminus of β-arrestin, the binding of β-arrestin to the receptor can be measured by BRET (Bioluminescence Resonance Energy Transfer).

The use of BRET has previously been limited by lack of sensitive instrumentation. Now assays have successfully been developed for several 7TM receptors using the multimode plate reader Mithras LB 940, BERTHOLD TECHNOLOGIES. This assay holds many advantages over other cell based functional screening assays. As the binding of β-arrestin to 7TM receptors is a universal process the assay can be used as a screening assay for virtually all 7TM receptors independent of their signaling pathway.

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BRET Principle

Renilla Luciferase (Rluc) emits light at 400 nm if brought into contact with the substrate DeepBlueC™. If one molecule (e.g. a receptor) labeled with Renilla Luciferase (Rluc) binds another molecule (e.g. β-arrestin) labeled with GFP^2 then an energy transfer will occur between Rluc and GFP^2 as the two molecules are brought into close proximity. The GFP^2 molecule will then emit light at a higher wave length (515 nm), this is BRET. Results are calculated as the ratio of GFP^2 light over Rluc light.

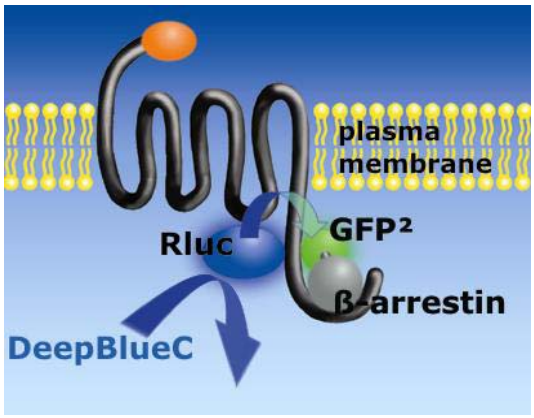

Figure 1: BRET principle

Mithras LB 940

The Mithras LB 940 is a multimode plate reader with a unique optical design (DOPS – Dedicated Optical Path System) to ensure optimized performance for the detection technologies implied. These are luminescence, BRET, fluorescence (top and bottom reading), absorbance, fluorescence polarization and AlphaScreen™. In addition accessory options, e.g. reagent injectors, temperature control and cooled PMT detection units are available.

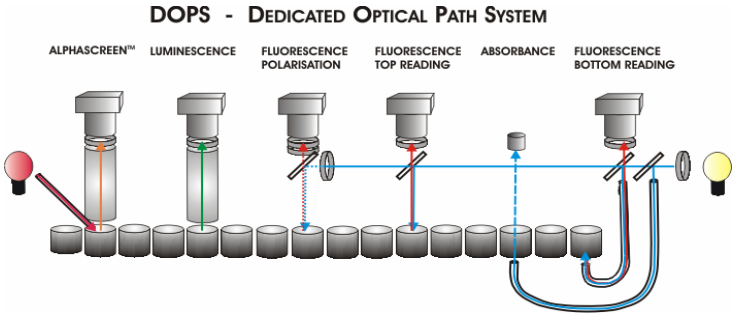

Figure 2: Dedicated Optical Path System and Mithras LB 940

Experimental Procedures

Assay protocol

48h before use, cells were transfected with β-arrestin-GFP[2] and the desired receptor-Rluc construct. The cells were then detached and re-suspended in D-PBS with 1000 mg/l L-Glucose to a density of 2x10[6] cells/ml. DeepBlueC™ was diluted to 50 µM in D-PBS with 1000 mg/l L-Glucose (light sensitive). 100 µl of cells were transferred to wells in a 96-well white OptiPlate plate and the plate was placed in the Mithras LB 940 instrument. 12 µl/well agonist were then injected by injector 1 and 10 µl DeepBlueC™/well were injected simultaneously by injector 2. 5 seconds following the injections the light output from the well was measured sequentially at 400 nm and 515 nm, and the 400/515 ratio calculated. For an antagonist assay, the (potential) antagonist was added to the wells before the plate was placed in the Mithras LB 940 instrument.

Instrument settings

Mithras LB 940 is operated through the Windows® PC software MikroWin 2000 which also serves as a data evaluation tool. With the standard configuration pre-set parameter files for BRET readings are supplied which can be modified according to one´s needs. A BRET assay involves two readings with the respective filters put in place.

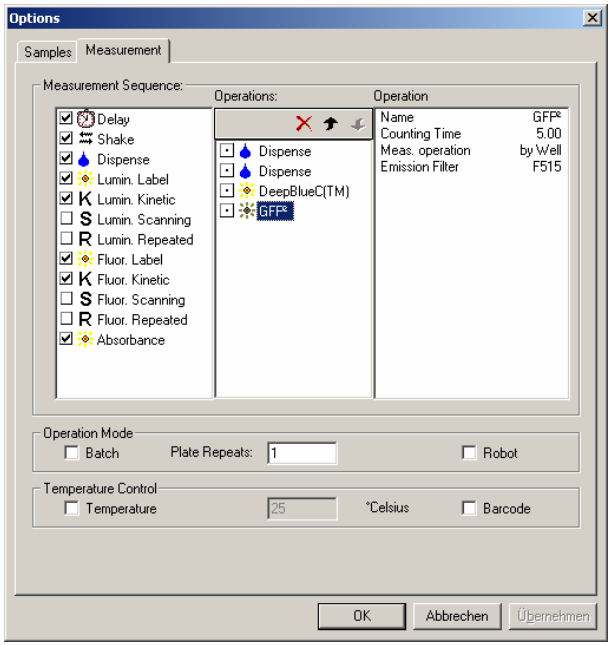

Figure 3: Operation sequence

Minimum data evaluation consists of the ratio calculation of the two readings.

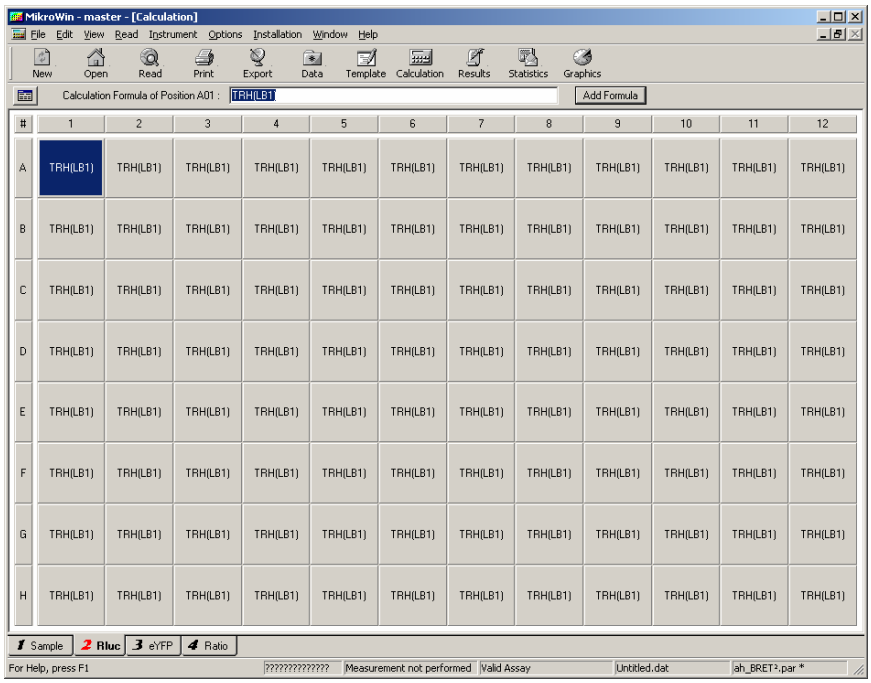

Figure 4: Calculation matrices. The expression *TRH(LB1)* refers to a default threshold setting (*TRH*) for documentation of detector overload and the abbreviation for the first label/reading (*LB1*).

Results

Agonist dose response assays

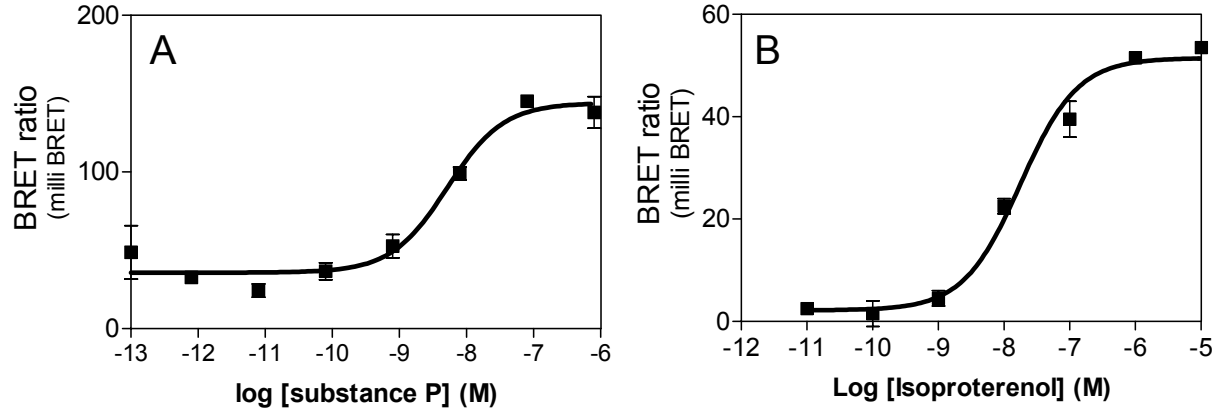

Figure 5. Agonist dose response curves for the NK1 (A) and β_2A receptor (B).

Increasing concentrations of the agonists Substance P and Isoproterenol were added to cells expressing Neurokinin 1 (NK1) receptor-Rluc/β-arrestin-GFP^2 (A) and β2 adrenergic (β_2A) receptor-Rluc/β-arrestin-GFP^2 (B) respectively. The receptor/β-arrestin interaction is measured as an increase in BRET ratio.

Antagonist dose response assays

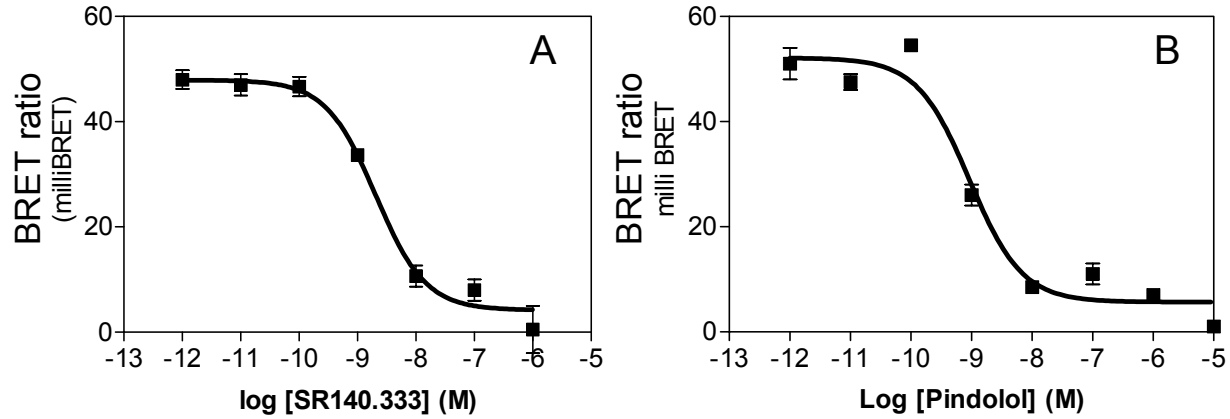

Figure 6. Antagonist dose response curves for the NK1 (A) and β_2A receptor (B).

Increasing concentration of the antagonists SR140.333 and Pindolol were added to cells expressing NK1 receptor-Rluc/β-arrestin-GFP^2 (A) and β_2A receptor-Rluc/β-arrestin-GFP^2 (B) respectively. Then agonist was injected, resulting in a final concentration of 20 nM (SP) or 1µM (Isoproterenol).

Agonist screening assays

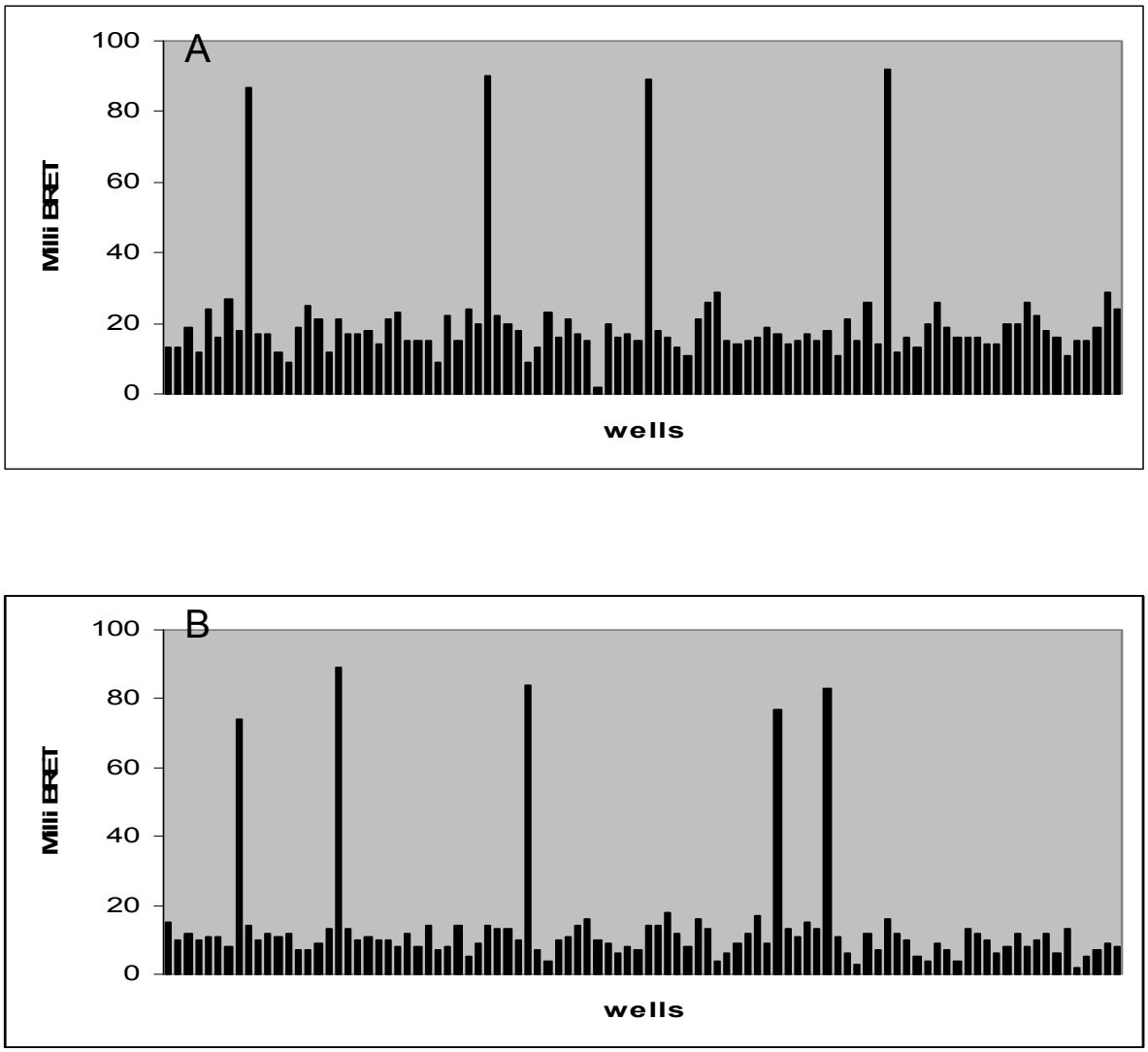

Figure 7. Agonist screening assays for the NK1 (A) and β_2A receptor (B).

The agonists Substance P and Isoproterenol were injected to selected wells in a 96 well plate containing cells expressing either NK1 receptor-Rluc/β-arrestin-GFP^2 (A) or β_2A receptor-Rluc/β-arrestin-GFP^2 (B). Substance P was injected to 4 wells in the NK1 plate and Isoproterenol was injected to 5 wells in the β_2A plate. The planted "hits" are clearly detected as the wells with the highest BRET ratio.

Antagonist screening assays

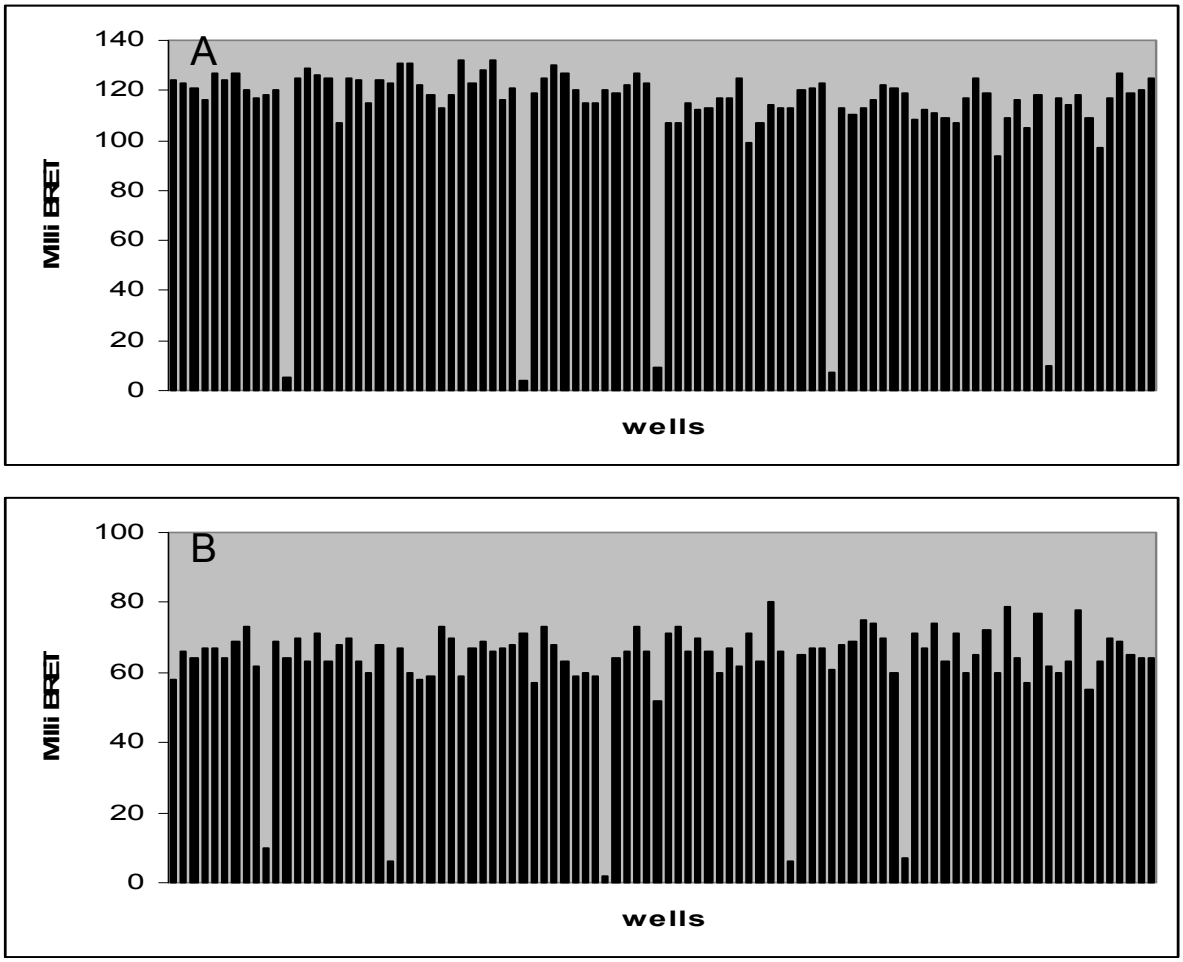

Figure 8. Antagonist screening assays for the NK1 (A) and β_2A receptor (B).

The antagonist SR140.333 was added to 5 wells in a 96 well plate containing cells expressing NK1 receptor-Rluc/β-arrestin-GFP^2 (A), and Pindolol was added to 5 wells in a 96 well plate containing cells expressing β_2A receptor-Rluc/β-arrestin-GFP^2 (B). The relevant agonists were then injected to all wells. The planted "hits" are clearly detected as the wells with the lowest BRET ratio

Conclusion

A functional BRET assays has successfully been developed for several 7TM receptors, two of which are described here, using the Mithras LB 940 from BERTHOLD TECHNOLOGIES. The Mithras LB 940 is well suited for both smaller assays e.g. dose response assays as well as larger assays such as the screening of full plates. The Mithras LB 940 was found to be more sensitive than any other readers offering BRET capability. Other plate readers do not perform BRET readings to satisfaction as they lack the ultimate sensitivity needed. This combined with the capability to inject samples is essential when running more than a few wells, as the substrate DeepBlueC™ burns out fast (in seconds) after addition to the well. The BRET technology described here is not limited to the study of receptor/β-arrestin interactions. We have successfully studied other protein-protein interactions such as receptor dimerisation using the Mithras LB 940 instrument.

Materials

- Cells: COS 7 cells transiently transfected with receptor-Rluc and β arrestin-GFP^2.
- Mithras LB 940, BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany.
- DNA: β-arrestin-GFP^2 and Rluc DNA was obtained from BioSignal Packard Inc, Montreal, Canada. The receptor-Rluc constructs were made using standard molecular biology techniques.
- White 96 well OptiPlates were obtained from Packard BioScience, Montreal, Canada.
- The substrate DeepBlueC™ was from BioSignal Packard Inc, Montreal, Canada. Standard medium, buffers, etc. was used from various manufactures.