

Aequorin bioluminescence assays on MicroBeta² LumiJET for studying Ca²⁺-coupled GPCRs and ion channels and ion channels

Introduction

Aequorin-based Ca²⁺ assays represent a new paradigm in drug discovery research for cell-based assays for Ca²⁺-coupled GPCRs and ion channels. In the aequorin assay, cells co-expressing apo-aequorin and the target receptor are first incubated with the co-factor coelenterazine in order to reconstitute the active aequorin enzyme.

Reconstitution of an active aequorin,

using native coelenterazine or its derivative coelenterazine h, yields an enzyme having a fast luminescent response to increasing calcium concentrations, and a high level of signal intensity. The aequorin photoprotein undergoes a bioluminescent reaction in the presence of calcium ions, producing a flash of light peaking at 469 nm. This wavelength correlates well with the maximum quantum efficiency of the PMTs used in the MicroBeta².

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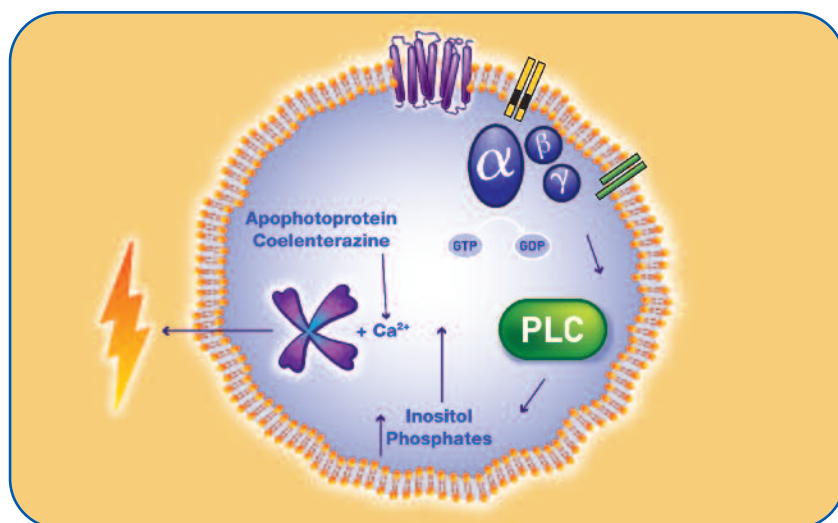


Figure 1: Principle of the method. Apo-aequorin is expressed at high level using a proprietary expression vector. Before the assay, cells are washed and loaded with coelenterazine. Cells are then dispensed into a plate with the tested compounds and the reaction is followed for 30 seconds. With compounds acting as agonists there will be a resultant emission of a light flash that can be measured with a luminescence reader.

The fast Ca^{2+} flux is usually measured in seconds and reagent injection is therefore imperative. Furthermore, the overall reaction may take twenty or more seconds to complete, resulting in processing times in excess of half an hour for each 96-well plate when counted on a conventional single detector luminometer. A 12-detector MicroBeta² LumiJET can reduce this throughput time by a factor of twelve, making this aequorin-based measurement of cellular Ca^{2+} an appropriate method for a HTS environment.

MicroBeta² is a multi-detector instrument designed for liquid scintillation (LS) or luminescence detection of samples in microplates, tubes or on filters. MicroBeta² has either 1, 2, 6 or 12 detectors and can count 24- and 96- and 384-well plate formats. Each detector in MicroBeta² is comprised of two photon multiplier tubes (PMTs), one above the sample and one below. In most cases, luminescence assays are counted with the upper PM tubes in solid white or black microplates. However, in the case of cell-based assays, they may be performed in clear bottomed plates, as the lower PMTs can give a higher signal.

MicroBeta² can be equipped with reagent injectors needed to perform aequorin assays. This model is called the MicroBeta² LumiJET. The performance of the MicroBeta² LumiJET is highly suitable for a wide range of radiolabel-based assays in addition to “flash and glow” type luminescence assays. With two injectors per well, the MicroBeta² LumiJET offers the ability run aequorin-based GPCR assays in a dual screening mode, providing both agonist and antagonist information about a compound.

To test performance of aequorin/ Ca^{2+} measurements in MicroBeta² LumiJET, agonist and antagonist assays in two cell lines were studied. AequoScreen[®] and AequoZen[®] FroZen cells stably expressing both mitochondria-targeted aequorin and a target GPCR were used in this study as described below.

Materials and Methods

Suspension cells assay

Measurements were performed with Histamine H₁ AequoScreen (PerkinElmer #ES-390-A) and Muscarinic M₅ AequoZen FroZen cell lines (PerkinElmer #ES-214-AF). Assays were performed in 384-well format with cell density of 5,000 or 6,000 cells per well for Histamine H₁ and Muscarinic M₅ assays, respectively. Cell harvesting, coelenterazine h (Invitrogen[®], #C 6780) loading and preparation were done according to instructions presented in the AequoScreen Starter Kit Manual.

Compound concentration series (20 μL /well) were diluted in 0.1 % BSA (Intergen, 3440-75) containing assay buffer (D-MEM/F-12, Invitrogen #11039) and prepared in white opaque Optiplate[™] 384-well microplates (PerkinElmer, #6007290). The cell suspension was dispensed on the ligands using the MicroBeta² LumiJET injectors. Injector tubing was rinsed (ethanol, water and assay buffer) and primed before injections.

Detection of agonist

Histamine (Sigma, #H7250) was used as an agonist for the Histamine H₁ cell lines and acetylcholine chloride (BioChemika, #A2661) for the Muscarinic M₅ cell line. The concentrations and dilutions series having eight replicates were prepared as instructed in the AequoScreen Starter Kit Manual. Emitted light was recorded kinetically (0.1 second measurement time) and integrated for 25 seconds.

Detection of antagonist

For the antagonist assay, cells were injected (30 μL) into the assay plate with antagonists (20 μL) using the MicroBeta² LumiJET. The antagonist dilution series with eight replicates was prepared as instructed in the AequoScreen Starter Kit Manual.

Antagonist used for the Histamine H₁ cell line was trans-triprolidine (Sigma, T6764) and N-Me-Scopolamine (S8502, Sigma) for the Muscarinic M₅ cell line. Agonist (histamine or acetylcholine) at a single concentration was injected (20 μL , final concentration 10 x EC₅₀) on the preincubated (50–55 min) of cells + antagonist and the emitted light was recorded kinetically (0.1 s measurement time) and integrated for 25 seconds.

Positive controls

Triton[®] X-100 (Fluka, #93420) at a 0.1% concentration in assay buffer was used to measure the receptor-independent cellular calcium response (cell membrane permeabilization) and acted as a positive control for the coelenterazine cell loading. Also ATP at 10 μM (ATPlite[™] ATP standard, PerkinElmer #6016736) was used as a positive control for the endogenous response within CHO-K₁ cells (purinergic P2Y receptor).

Determination of Z' factor

To evaluate the Z' factor for different Aequorin assays, a separate Z' prime plate was prepared containing assay buffer, agonist and antagonist wells (in 60 replicates). The concentrations for agonists and antagonists in wells were 10 x EC₅₀/IC₅₀. Cells were injected into wells with MicroBeta² LumiJET, incubated for ~1 h and agonist (10 x EC₅₀ concentrations) was added using the second injector module of MicroBeta² LumiJET.

Normalization

The performances of individual photon multiplier tubes (PMT) are never completely identical. Before counting unknown samples a calibration procedure is needed to determine the relative efficiency of each PMT. This procedure is called detector normalization. As the emission light produced by aequorin is in the blue range, optimal normalization results are obtained with a light source in a corresponding

wavelength range is used to match each of the PMTs together. The normalization can be performed by using a stable glow luminescence sample with the injector module in place. Once normalization has been completed, the relevant data is stored for future use and can be linked with a counting protocol. The background of each PMT is also determined in the normalization run and it is automatically subtracted from sample responses.

Results and Discussion

Aequorin assays in MicroBeta² LumijET were performed in a kinetic mode to study the kinetic signal response, as well as the total signal output. The resolution of 0.1 s from the flash luminescence reaction is high enough to discriminate the signal peak differences of variable compound concentra-

tions (Figure 2). For agonist and antagonist experiments the sum of all 0.1s kinetic points

over a time of 25 seconds was used as a response.

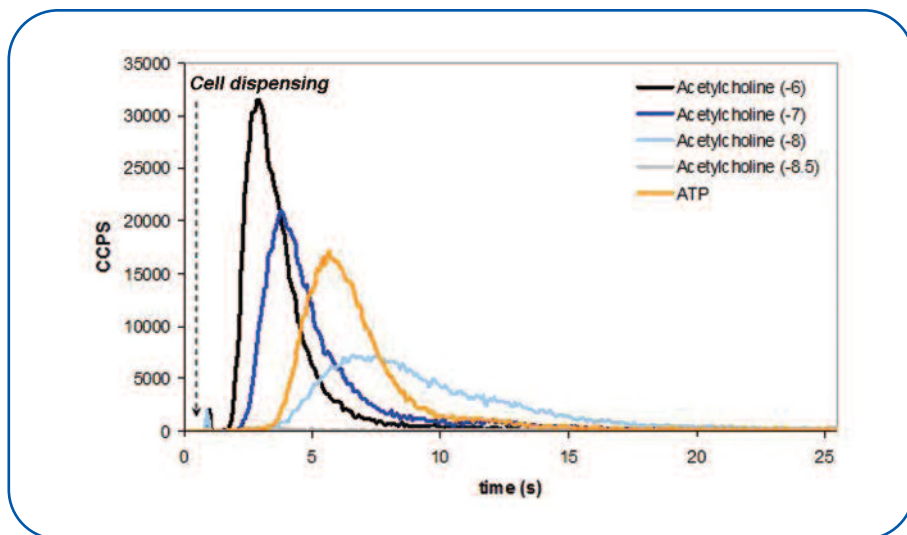


Figure 2. Kinetic graphs of the cellular response to Muscarinic M₅ receptor stimulation by an agonist (acetylcholine). The response to 10 μM ATP, acting on an endogenous P2Y receptor present in CHO cells is also presented. Overlaid kinetic graphs presented with Microsoft Office Excel.

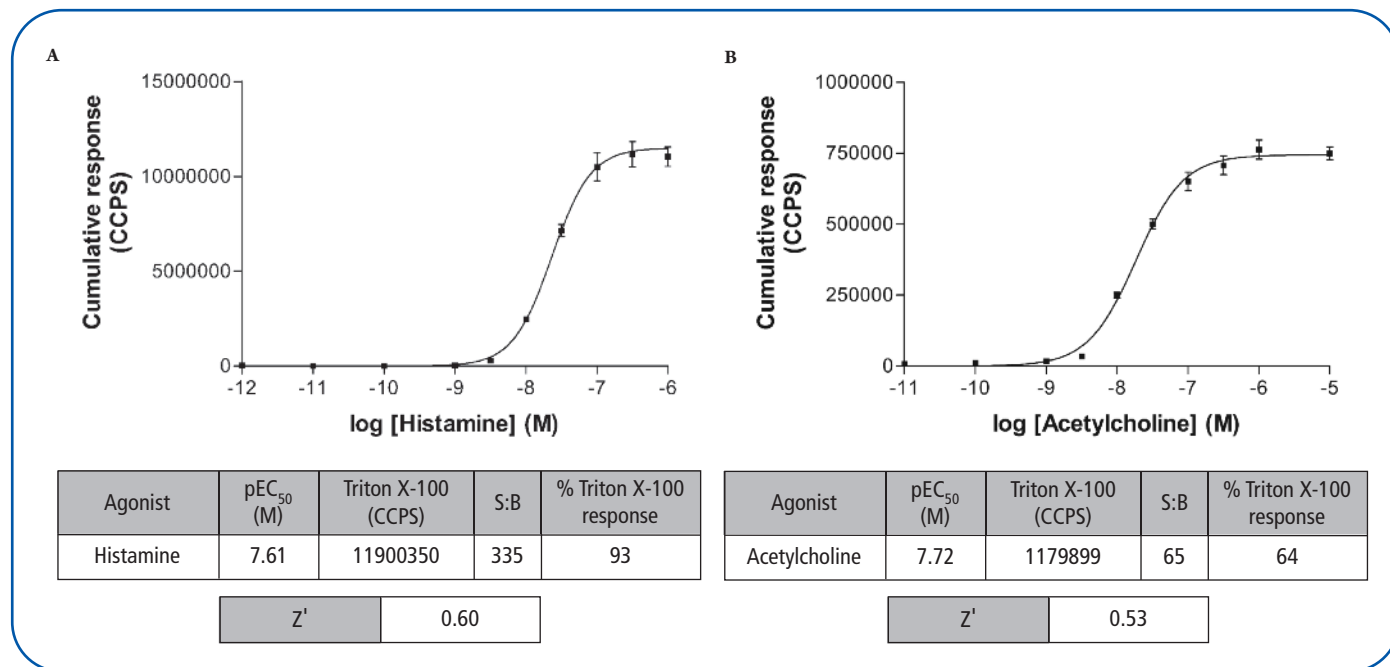


Figure 3: The agonist dose-response curves and EC₅₀ values for A) histamine on AequoScreen histamine H₁ cell line and B) for acetylcholine on M₅ Muscarinic FroZen cell line. Curve fitting, error bars (SD) plotted with GraphPad Prism®.

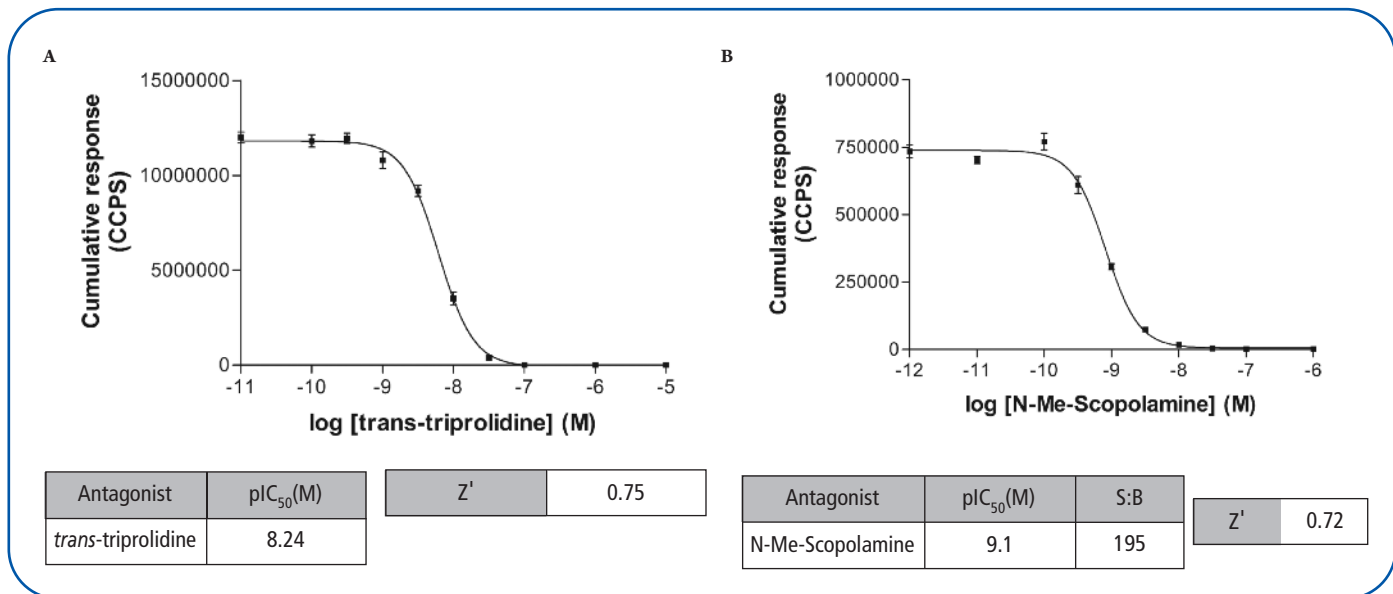


Figure 4. A) Inhibition by trans-triprolidine of the stimulation of the H₁ receptor by histamine. B) N-Me-Scopolamine inhibited the stimulation of M₅ receptor by acetylcholine. Both antagonists prevented, in a dose dependent manner, the activation of their corresponding receptors by an agonist. Curve fitting, error bars (SD) plotted with GraphPad Prism®.

The agonist and antagonist assay dose response curves (Figures 3 and 4) for both used cell lines demonstrate the quality of data produced by the MicroBeta² LumiJET. Both the EC₅₀ and IC₅₀ values are in accordance with previously defined values and together with the Z' > 0.5 both in the high (Histamine H₁) and low (Muscarinic M₅) signal producing cells lines shows that AequoScreen assays can be run successfully with 384-well format using MicroBeta² LumiJET and with relatively low cell numbers.

Summary

The MicroBeta² LumiJET introduces new features and capabilities enabling a variety of assays to be run with high throughput and ease.

The aequorin assays that are presented here were run with a 12-detector MicroBeta² model with two injectors per well. This model is ideal for flash type luminescence assays that need high throughput in combination with high sensitivity luminescence detection. It provides all the tools

required for studying calcium-related GPCR and ion channel assays.

With MicroBeta², the GPCR assays can be run in single mode to determine agonist or antagonist effect of a molecule or in dual screen mode to determine both agonist and antagonist response for one sample plate. In a 384-well dual screen, the plate is run sequentially, generating approximately 20 minutes effective time for a possible antagonist.



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