PhotoScreen™ Luminescent Calcium Assays: Applications on the LumiLux® and FLIPR^{TETRA}®

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Abstract

Ca^{2+}-activated photoproteins are important tools for analyzing all aspects of Ca^{2+}-mediated signal transduction processes in mammalian cells. One of their characteristics is the immediate photon release (flash luminescence) upon Ca^{2+} binding to the coelenterazine-photoprotein complex, which makes this system extremely useful for studying rapid receptor-ligand interactions or fast acting ion channels involving Ca^{2+} mobilization.

PhotoScreen™ cell lines express the Photina® or i-Photina® proteins, two extremely bright photoproteins generated at Axxam S.p.A. and optimized for HTS campaigns. PhotoScreen™ assays allows the detection of rapid increases in intracellular Ca^{2+} concentration with almost no background activity and a consequent high signal-to-background ratio and broad range of detection sensitivity.

Data generated on the LumiLux® Cellular Screening Platform and FLIPR^{TETRA}® system with PhotoScreen™ cell lines overexpressing different classes of targets will be presented.

Background information:

Photoprotein-mediated flash luminescence is a valuable tool for studying any intracellular calcium increase triggered by receptors activation. Several different photoproteins have been used for this purpose, and aequorin has been one of the most known so far.

A number of Photina® or i-Photina® parental cell lines have been created and used for generating double stable PhotoScreen recombinant cell lines co-expressing a target gene of interest and the most suited photoprotein for calcium detection. In this work, the performance of the stable CHO PhotoScreen A3 receptor cell line (PhotoScreen Starter Kit; cat. number AX-001-PCF) and HEK PhotoScreen TRPA1 (cat. number AX-004-PCL) ion channel cell line were evaluated on the LumiLux® and FLIPR^{TETRA}® instruments.

Methods

Cloning

Human TRPA1 (GeneID 8989) and human A_3 receptor (GeneID 140) cDNAs were amplified by RT-PCR from human cDNA. The PCR products were cloned into pcDNA3.1 (Invitrogen Corp.) and sequence was confirmed by DNA sequencing.

For the A_3 receptor, a fusion protein between A_3 and $G_{\alpha 16}$ was created in order to switch the natural signaling of the A_3 receptor to the phospholipase C/calcium pathway.

Cell culture

CHO K1 permanently expressing the Ca^{2+}-activated photoprotein i-Photina® and the A_3 gene were maintained in Dulbecco's MEM/Nutrient Mix F12 supplemented with UltraGlutamine, sodium pyruvate, HEPES, sodium bicarbonate, penicillin/streptomycin, 10% FBS and 1 mg/mL G418. HEK-293 permanently expressing the Ca^{2+} activated photoprotein Photina® and the TRPA1 gene were maintained in Minimum Essential Medium with Earle's salts supplemented with UltraGlutamine, Penicillin/Streptomycin, 10% FBS and 0.4 mg/mL G418.

Cell transfection and clone selection

After transfection by electroporation with the A_3 or TRPA1 cDNA, cells were grown in the presence of the selective agent G418 and underwent two rounds of limiting dilutions.

Best responder clones were selected based on the overall content of photoprotein and on the basis of the best functional response of the specific transfected gene.

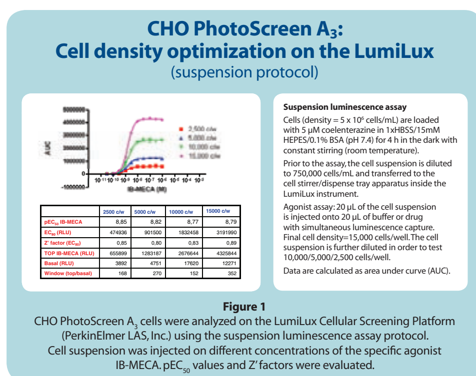

Figure 1

CHO PhotoScreen A_3 cells were analyzed on the LumiLux Cellular Screening Platform (PerkinElmer LAS, Inc.) using the suspension luminescence assay protocol. Cell suspension was injected on different concentrations of the specific agonist IB-MECA. pEC_{50} values and Z' factors were evaluated.

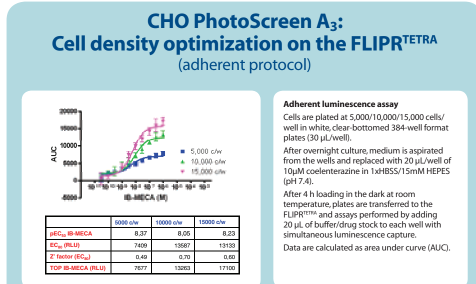

Figure 2

CHO PhotoScreen A_3 cells were analyzed on the FLIPR^{TETRA} (Molecular Devices) upon injection of increasing concentrations of the specific agonist IB-MECA. pEC_{50} values and Z' factors were evaluated.

FLIPR^{TETRA}® analysis

Cells were seeded in white walled clear-bottom 384-well plates at the indicated density in growth media depleted of G418 and allowed to adhere for 24 hours.

Cells were loaded with 10 μM native coelenterazine solubilized in HBSS/15 mM HEPES buffer (pH 7.4) for 4 hours at room temperature. Luminescence was monitored following compound injection using the FLIPR^{TETRA}® instrument.

Luminescence detection with the LumiLux®

Cells were loaded in batch with 5 μM coelenterazine at a density of 5 x 10^6 cells/mL in 1x HBSS/15 mM HEPES/0.1% BSA (pH 7.4) for 4 hours in the dark with constant stirring at room temperature. Prior to the assay, cell suspensions were diluted at 750,000 cells/mL and transferred to the cell stirrer/dispense tray apparatus inside the LumiLux® instrument.

They were then dispensed at the indicated density in black-walled clear-bottom 384-well plates. Luminescence was monitored following compound injection using the LumiLux® instrument.

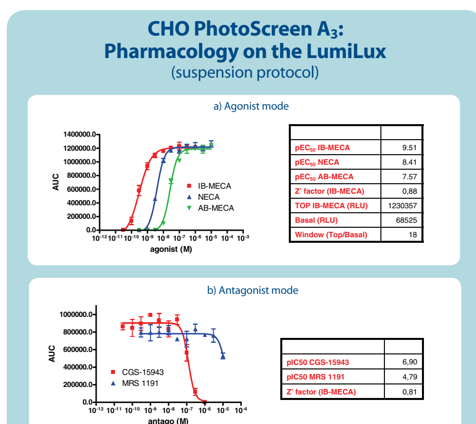

Figure 3

CHO PhotoScreen A_3 cells were analyzed on the LumiLux Cellular Screening Platform using the suspension luminescence assay protocol as described in Fig.1. The cell suspension (5,000 cells/well) was injected on:

3a) different concentrations of three A_3 agonists (IB-MECA, NECA and AB-MECA). pEC_{50} values and Z' factors were evaluated.

3b) different concentrations of two antagonists (MRS 1191 and CGS-15943). 10 min later IB-MECA at EC_{80} concentration was injected. pIC_{50} values and Z' factors were evaluated.

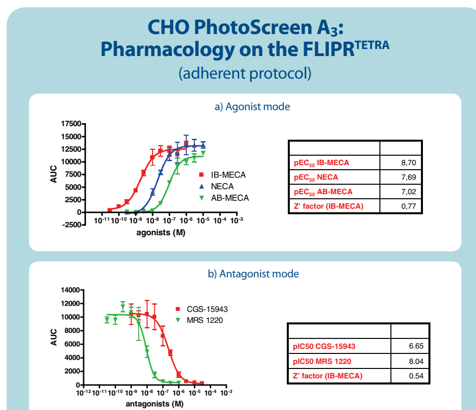

Figure 4

CHO PhotoScreen A_3 cells were analyzed on the FLIPR^{TETRA} according to the protocol described in Fig.2. Cells at a density of 5,000 cells/well were tested 24 h after seeding upon injection of

4a) increasing concentrations of specific agonists (IB-MECA, NECA, AB-MECA). pEC_{50} values and Z' factors were evaluated.

4b) increasing concentrations of antagonists (MRS 1191 and CGS-15943). 10 min later IB-MECA at EC_{80} concentration was injected. pIC_{50} values and Z' factors were evaluated.

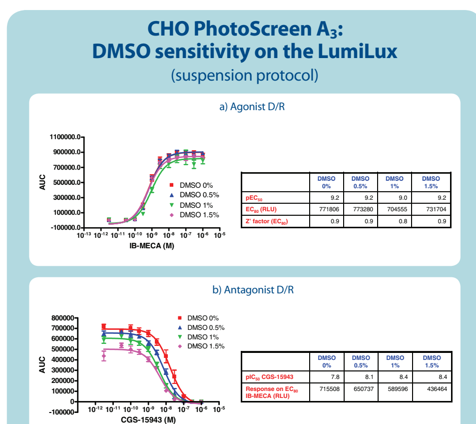

Figure 5

CHO PhotoScreen A_3 cells were analyzed on the LumiLux Cellular Screening Platform using the suspension luminescence assay protocol.

5a) The cell suspension (5,000 cells/well) was injected on different concentrations of the specific agonist IB-MECA in presence of different DMSO concentrations. pEC_{50} values and Z' factors were evaluated.

5b) The cell suspension (5,000 cells/well) was injected on different concentrations of DMSO. 10 min later, a solution of IB-MECA (EC_{80}) was injected to the wells. pIC_{50} values were evaluated.

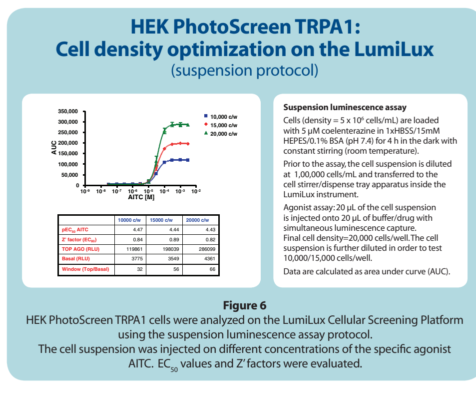

Figure 6

HEK PhotoScreen TRPA1 cells were analyzed on the LumiLux Cellular Screening Platform using the suspension luminescence assay protocol. The cell suspension was injected on different concentrations of the specific agonist AITC. EC_{50} values and Z' factors were evaluated.

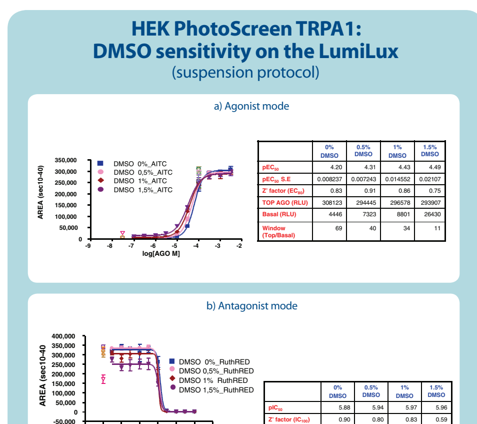

Figure 7

HEK PhotoScreen TRPA1 cells were analyzed on the LumiLux Cellular Screening Platform using the suspension luminescence assay protocol. The cell suspension (20,000 cells/well) was injected on different concentrations of the specific agonist AITC (agonist mode) or Ruthenium Red (antagonist mode) in presence of different DMSO concentrations. EC_{50}/IC_{50} values and Z' factors were evaluated.

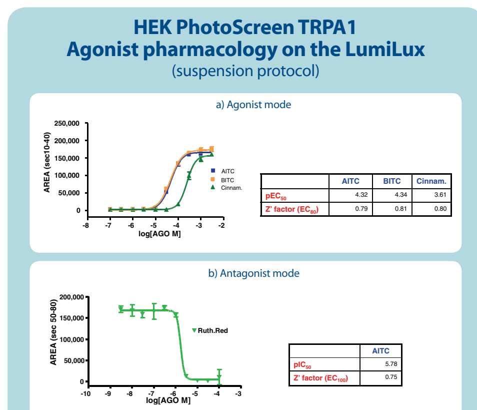

Figure 8

HEK PhotoScreen TRPA1 cells were analyzed on the LumiLux Cellular Screening Platform using suspension luminescence assay protocol. The cell suspension (20,000 cells/well) was injected on:

8a) different concentrations of three TRPA1 agonists (AITC, BITC, Cynnamaldheide). EC_{50} values and Z' factors were evaluated.

8b) different concentrations of the TRPA1 antagonist Ruthenium Red. 10 min later, 20 μL/well of AITC at 80 μM, final concentration, was injected. IC_{50} values and Z' factors were evaluated.

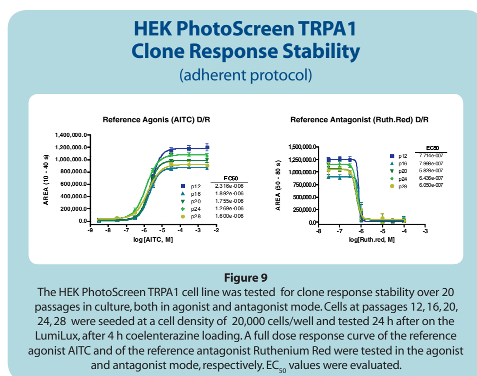

Figure 9

The HEK PhotoScreen TRPA1 cell line was tested for clone response stability over 20 passages in culture, both in agonist and antagonist mode. Cells at passages 12, 16, 20, 24, 28 were seeded at a cell density of 20,000 cells/well and tested 24 h after on the LumiLux, after 4 h coelenterazine loading. A full dose response curve of the reference agonist AITC and of the reference antagonist Ruthenium Red were tested in the agonist and antagonist mode, respectively. EC_{50} values were evaluated.

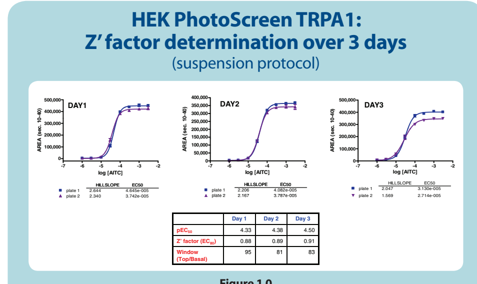

Figure 10

The HEK PhotoScreen TRPA1 cell line was analyzed on the LumiLux Cellular Screening Platform using the suspension luminescence assay protocol. Cells were incubated as described in Figure 6, diluted to a final concentration of 20000 cells/well, let equilibrate for 30 min, and then injected on 100 μM AITC (EC_{90}). The same experiment was repeated on 3 different days.

Concluding remarks

1. Both the CHO PhotoScreen™ A_3 cell line (from the PhotoScreen™ Starter Kit) and HEK PhotoScreen™ TRPA1 cell display very strong, stable and reproducible luminescence signal upon agonist stimulation.

2. Receptors functionality was demonstrated by monitoring flash luminescence emitted by the Ca^{2+}-activated Photina® and i-Photina® photoproteins on both FLIPR^{TETRA}® and LumiLux® instruments.

3. We have verified the TRPA1 channel electrophysiological properties by patch clamp experiments.

4. We have determined the EC_{50} and IC_{50} values of different agonists and antagonists and we have obtained reproducible results, independently on the detection system applied.

5. We have assessed the assay robustness and data reproducibility by determining the Z' values for agonists as well as antagonists.

6. We have assessed the sensitivity to DMSO of the assays to fully validate screening processes, suitable for the identification of both GPCR and ion channels.