Running an Aequorin Cell-Based Luminescent Assay on the FLIPR$^{TETRA®}$

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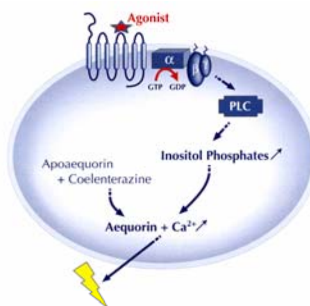
1 Introduction



Aequorin-based Ca^{2+} assays represent a new paradigm in drug discovery research for Ca^{2+}-coupled GPCRs and ion channels cell-based assays. The inherent limitations of fluorescent dye-based screening result in lower throughput and higher resource expenditure in comparison to the aequorin assay. Furthermore, in a HTS setting the fluorescence assay approach is prone to interference from autofluorescent compounds resulting in a large percentage of false positive or false negative hits in agonist and antagonist screens respectively. Dedicated, highly sensitive readers such as the LumiLux® (PerkinElmer), the MicroBetaJet® (PerkinElmer), the FDSS (Hamamatsu Photonics) and the Cybi®Lumax (Cybio) have already been in use for several years to perform drug discovery research using aequorin assays. Here, we show that selected aequorin cell lines generating intense luminescent signal allow comfortable detection with a less sensitive, non-luminescence dedicated reader, i.e. the FLIPR$^{TETRA®}$ (Molecular Devices). The FLIPR$^{TETRA®}$ was built for fluorescent signal detection, but by putting off the excitation light, can also be used for strong luminescent signal detection. We show several examples of aequorin signal detection with the FLIPR$^{TETRA®}$, generating high quality data, with Z' values compatible for HTS use of Aequoscreen® assay on the FLIPR$^{TETRA®}$.

2 Reagents

Double transfected **aequorin cell lines**, stably expressing both mitochondria-targeted aequorin and a GPCR were used in this study: Histamine H_1 AequoScreen® cell line (PerkinElmer # ES-390-A), Vasopressin V_{1B} AequoScreen®; cell line (PerkinElmer # ES-362-A), Vasoactive Intestinal Peptide $VPAC_1$ AequoScreen® cell line (PerkinElmer # ES-273-A). In addition, the CHO-K1 (+Gα16) AequoScreen® Parental cell line (PerkinElmer # ES-000-A24) was also used, using ATP as an agonist of the endogenous P2Y receptor.

Assay Buffer was DMEM/Ham's F12 culture medium (with 15 mM HEPES, L-glutamine, without phenol red; Invitrogen Cat n°11039) + 0.1% protease-free BSA. **Coelenterazine h** came from Promega (Cat n° S2011): 1 mM stock solution in methanol. **Digitonin** came from Sigma (Cat n°37006): 50 mM stock solution in DMSO.

3 AequoScreen® assay

Adherent cells assay: For FLIPR$^{TETRA®}$ measurements, cells were tested mainly in adherent mode (10 000 cells/well unless otherwise indicated), but some suspension cell assays were also performed. Cells (frozen vial) were thawed and plated in TC-treated assay plates (in Ham'sF12 with 10% serum, no antibiotics) and left in the incubator overnight (37°C, 5% CO_2). The next day, medium was removed by plate overthrow and tapping on a paper towel, then 20 µL/well of Assay Buffer containing 10 µM coelenterazine h was added to the cells and plates were incubated for 4h at RT° in the dark. Ligands (20µL/well), diluted in Assay Buffer, were dispensed on the cells using the FLIPR®. We used black, clear bottom 384-well assay plates as we observed a background signal that was variable from well to well when using white assay plates (not shown).

Suspension cells assay: For LumiLux® measurements, cells were tested in suspension mode (5 000 cells/well). Cells (frozen vial) were thawed and resuspended in 10-ml of assay buffer containing 5 µM coelenterazine h. This cell suspension was put in a 10-ml Falcon tube, fixed onto a rotating wheel and incubated for 4 h or overnight at RT° in the dark (8 rpm; 45° angle). Cells were diluted with Assay Buffer to 5 000 cells/20 µL. Ligands (20µL/well), diluted in Assay Buffer, were prepared in black, clear bottom assay plates, and the cell suspension was dispensed on the ligands using the LumiLux®.

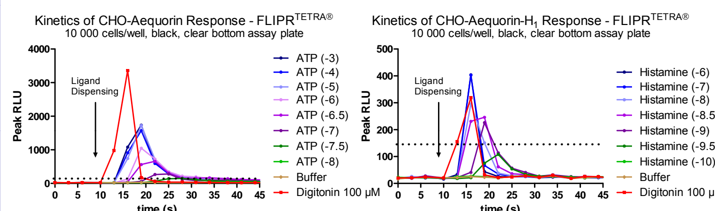
Digitonin at a final concentration of 100 µM diluted in Assay Buffer was used to measure the receptor-independent cellular calcium response (cell membrane permeabilization).

The "**Top/digitonin**" response (expressed in percentage) is the ratio between the maximal response to the ligand of the receptor (Top) and the digitonin response (Digitonin), which is indicative of the aequorin content of the cells.

FLIPR$^{TETRA®}$ settings: For using the FLIPR$^{TETRA®}$ in luminescence mode, the excitation light was disabled (select "Exc.WLength: NONE" in the software), a gain of 240 and an exposure time of 3 seconds were used. 20 µL of ligands were dispensed form the source plate 1 to the read plate containing the coelenterazine-loaded cells. 33 intervals of 3.1 seconds were read, 3 of them being read before ligand dispensing.

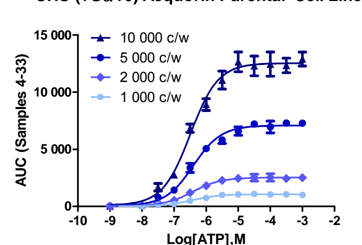
Area under the curve (AUC) was calculated with the ScreenWorks™ software, using the third interval for background subtraction ("subtract bias based on sample 3").

4 Kinetic measurement of Aequorin signal on the FLIPR$^{TETRA®}$

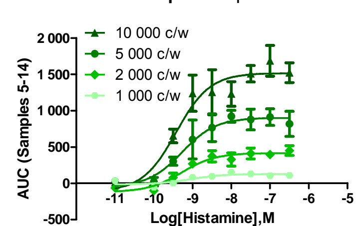


Using 3 second intervals, kinetics of the emission of light can be measured by the FLIPR$^{TETRA®}$. The kinetics of the responses of 2 AequoScreen® cell lines are shown. The signal intensity was well above 150 Peak RLU (dashed line), which is an acceptable threshold limit.

5 Number of cells per well - sensitivity



	pEC_{50}	Z' (EC_{100} Ago vs Buffer)
10 000 c/w	6.49	0.85
5 000 c/w	6.4	0.94
2 000 c/w	6.42	0.47
1 000 c/w	6.45	0.61

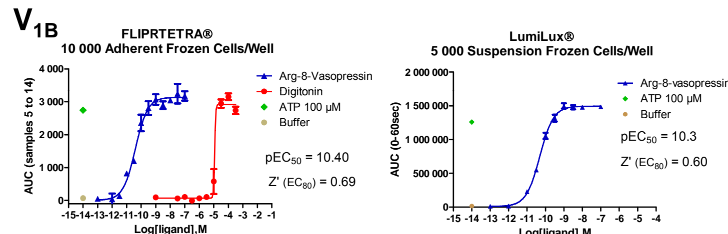


	pEC_{50}	Z' (EC_{100} Ago vs Buffer)
10 000 c/w	9.42	0.66
5 000 c/w	9.27	0.85
2 000 c/w	9.28	0.5
1 000 c/w	9.05	< 0

Increasing cell numbers per well were used to assess the sensitivity of the FLIPR$^{TETRA®}$ in relation with the brightness of the cell lines. EC_{50} values were unchanged by varying the cell density. The CHO (+Gα16) Aequorin parental cell line was brighter than the H_1 cell line, and yielded acceptable values even when using 1,000 cells/well. For the H_1 cell line, at least 2,000 cells/well were needed to get sufficient signal intensity.

6A Pharmacology (FLIPR$^{TETRA®}$ vs LumiLux®)

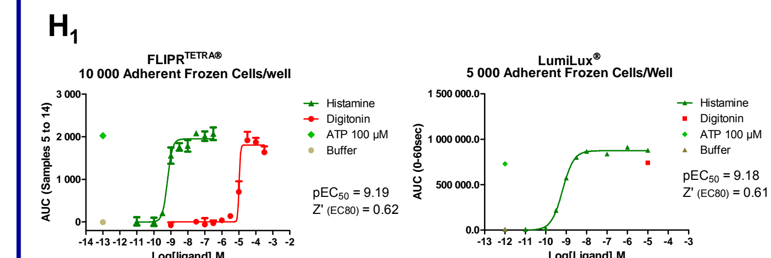
V_{1B}



CHO-Aequorin-Vasopressin $_{1B}$ receptor cells were analyzed on the FLIPR$^{TETRA®}$ and on the LumiLux® Cellular Screening Platform using the adherent (FLIPR®) and suspension (LumiLux®) luminescence assay protocols. EC_{50} values obtained on both readers and in both assay modes were equivalent.

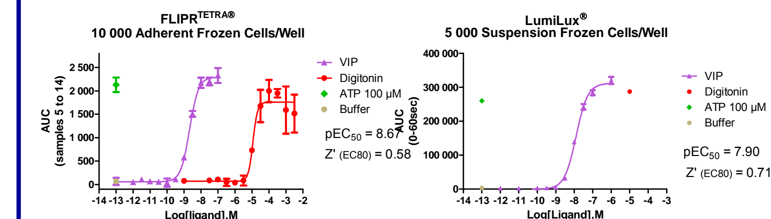
6B Pharmacology (FLIPR$^{TETRA®}$ vs LumiLux®)

H_1



CHO-Aequorin Histamine H_1 receptor AequoScreen® cells were analyzed on the FLIPR$^{TETRA®}$ and on the LumiLux® Cellular Screening Platform using the adherent luminescence assay protocols. EC_{50} values obtained on both readers and in both assay modes were equivalent.

$VPAC_1$



CHO-Aequorin Vasoactive Intestinal Peptide $VPAC_1$ receptor AequoScreen® cells were analyzed on the FLIPR$^{TETRA®}$ and on the LumiLux® Cellular Screening Platform using the adherent (FLIPR®) and suspension (LumiLux®) luminescence assay protocols. EC_{50} values obtained on both readers and in both assay modes were equivalent.

7 Conclusion

We have shown here that signal intensity for the selected catalog aequorin cell lines is strong enough to allow its measurement by the non-luminescence dedicated FLIPR$^{TETRA®}$. In particular, signal intensity of the CHO (+Gα16) aequorin parental cell line was very high, and thus will undoubtedly generate double-transfectant cell lines that will be suitable to use on the FLIPR$^{TETRA®}$. Pharmacology and Z' values observed are fully compatible with the use of these cell lines for HTS or profiling studies.

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