

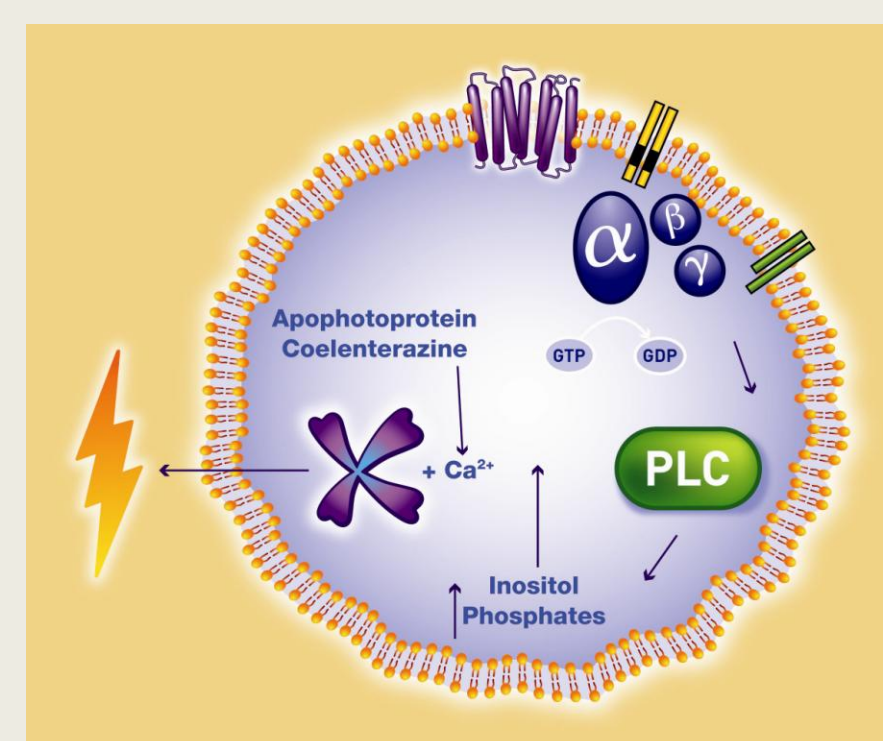
1 Introduction

Aequorin-based Ca^{2+} assays represent a new paradigm in cell-based high throughput screening (HTS) Ca^{2+} -coupled GPCRs and ion channels. Besides not being affected by autofluorescent compounds, the AequoScreen® methodology also presents advantages in terms of improved quality (Z' , window and hit confirmation rate), easiness of assay implementation, and increased throughput. Although the traditional fluorescent dye-based Ca^{2+} assays fundamentally measure the same intracellular event as aequorin, when looking at the kinetics of the response to agonist stimulation, signal decrease is faster in the aequorin assay compared to the Fluo-4 assay. On one hand, due to slow dissociation of Ca^{2+} from the fluorescent dye, kinetics in the fluorescent assay are extended compared to the real calcium flow. On the other hand, due to consumption of aequorin substrate during the measurement, the shape of the signal decrease in the luminescent assay is sharper than the true calcium decrease. As a result of these technical features, the difference between the maximal and minimal signal intensity (Max-Min or Peak value) is the preferred parameter used in the fluorescent assay, whereas the integration of the signal (Area Under the Curve, AUC) is more adequate in the luminescent assay. However, because of the historical spread of the fluorescent calcium assays, there is a natural tendency to use the "Max-Min" method to analyze Aequorin data.

In this work, we present how pharmacological parameters extracted from aequorin experimental results, such as EC_{50} , IC_{50} values and agonist efficacy, can differ according to the data processing method, i.e. "Max-Min" method vs "AUC" method.

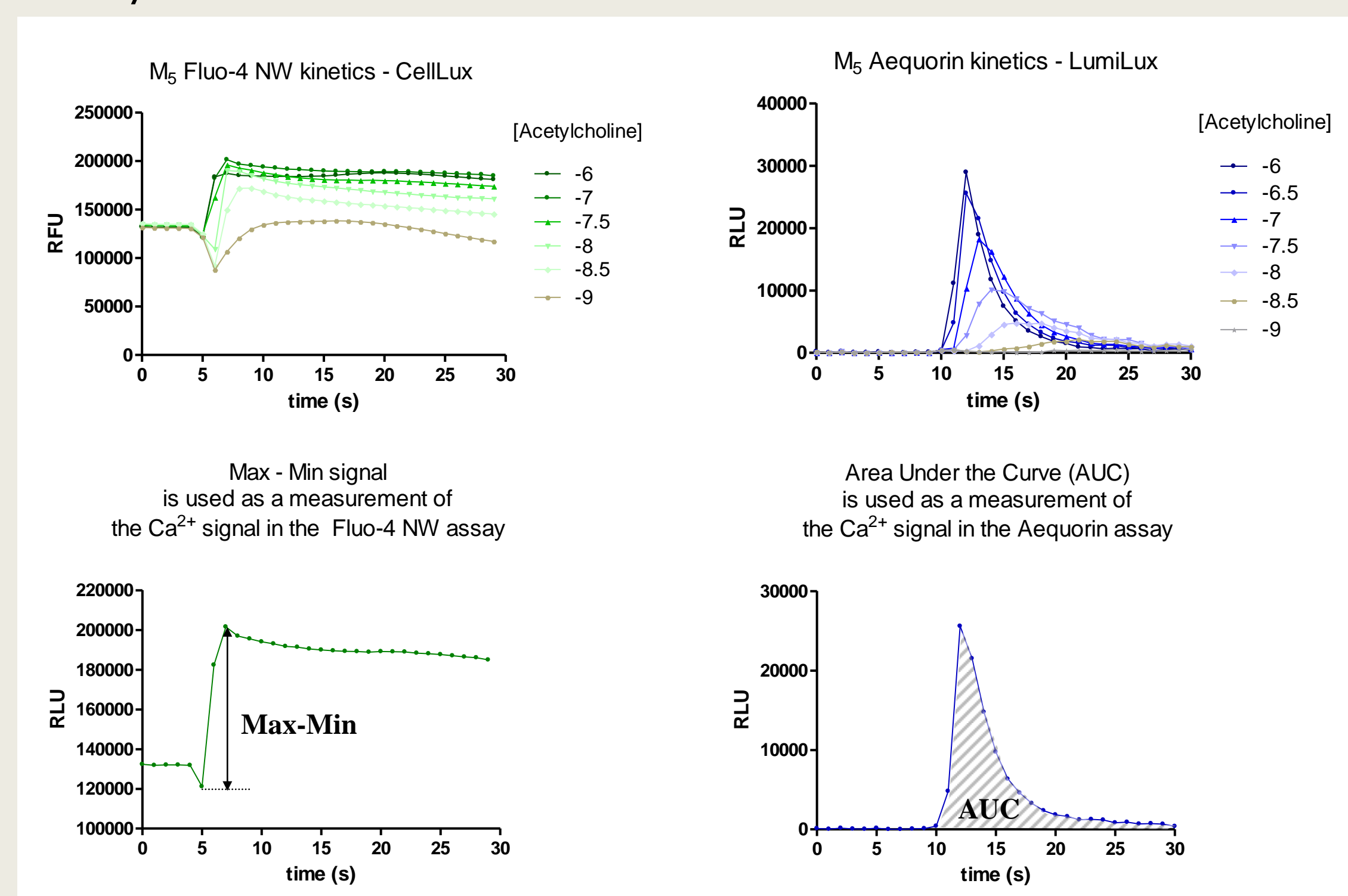
2 Principle of the AequoScreen® Technology

Cells are incubated with coelenterazine, the co-factor of aequorin. Coelenterazine enters the cell and combines with apoaequorin to form active aequorin. Agonist stimulation of the G-protein coupled receptor leads to release of Ca^{2+} from intracellular stores, therefore increasing cytoplasmic free Ca^{2+} concentration. This Ca^{2+} increase leads to the activation of the catalytic activity of aequorin, which oxidizes the coelenterazine substrate to produce CO_2 and a flash of light at 466 nm. When working with GPCRs, aequorin is targeted in mitochondria as it increases the signal intensity.



3 Kinetics of the signal in luminescence and fluorescence assays

Signal decrease is faster in the aequorin assay compared to the Fluo-4 assay. Due to slow dissociation of Ca^{2+} from the fluorescent dye, kinetics in the fluorescent assay are extended compared to the real calcium flow. On the contrary, due to consumption of aequorin substrate during the measurement, the shape of the signal decrease in the luminescent assay is sharper than the real calcium decrease. As a result of these features, the difference between the maximal and minimal signal intensity is the parameter used in the fluorescent assay, whereas the integration of the signal (Area under the Curve, AUC) is used in the luminescent assay. However, past users of the fluo-4 assay tend to use the Peak Value ("Max-Min") method in the aequorin assay as well.



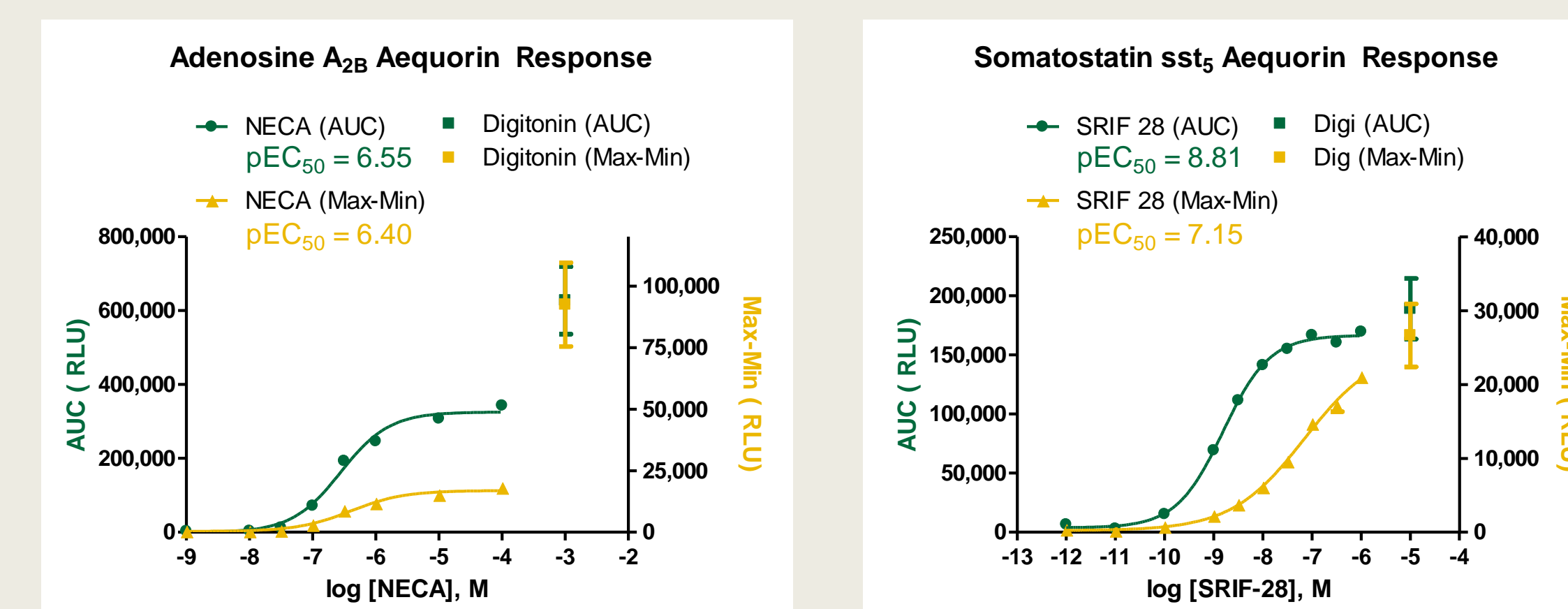
Kinetics of Fluorescent Calcium and AequoScreen® assays performed on the same AequoScreen® cell line expressing the muscarinic M_3 receptor.

4 pEC_{50} Values are Higher When Using "AUC" rather than "Max-Min"

In order to determine the impact of the data processing method on the pharmacological parameters, data from agonist and antagonist experiments performed on 100 stable AequoScreen® cell lines co-expressing a GPCR were analyzed using the "AUC" or the "Max-Min" methods. The agonist affinity values were more affected than the antagonist assay values. The "AUC" method yielded the highest Z' values.

Agonist Assays (100 receptors analyzed, average values)	AUC	Max-Min
ΔpEC_{50} (mean \pm SD)		1.09 \pm 0.56 lower value (right-shift) when using Max-Min compared to AUC
Z' (mean \pm SD)	0.59 \pm 0.19	0.46 \pm 0.25
Window (Highest Agonist Response/Buffer)	39	85
Hill Slope	1.23	0.73
TOP (graphpad fitting) / Digitonin	78%	110%
Highest Agonist Response/Digi	75%	88%
Curves having at least 3 experimental points at the upper plateau	79%	30%

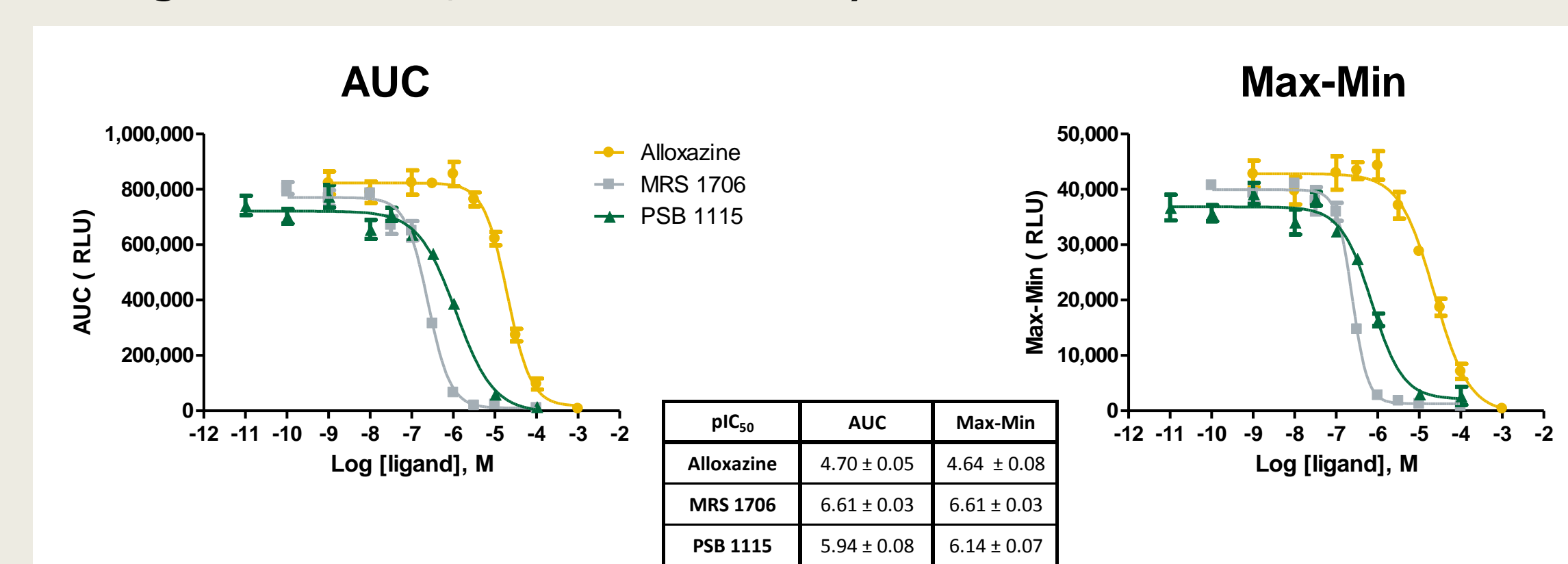
Antagonist Assays (7 receptors analyzed)	AUC	Max-Min
ΔpIC_{50} (mean \pm SD)		0.22 \pm 0.37 higher value (left-shift) when using Max-Min compared to AUC
Hill Slope	-1.35	-1.24



Impact of analysis method varies according to the receptor
Same data analyzed in AUC or Max-Min mode for 2 receptors

5 Rank Order of Potency is not Changed by the Analysis Method

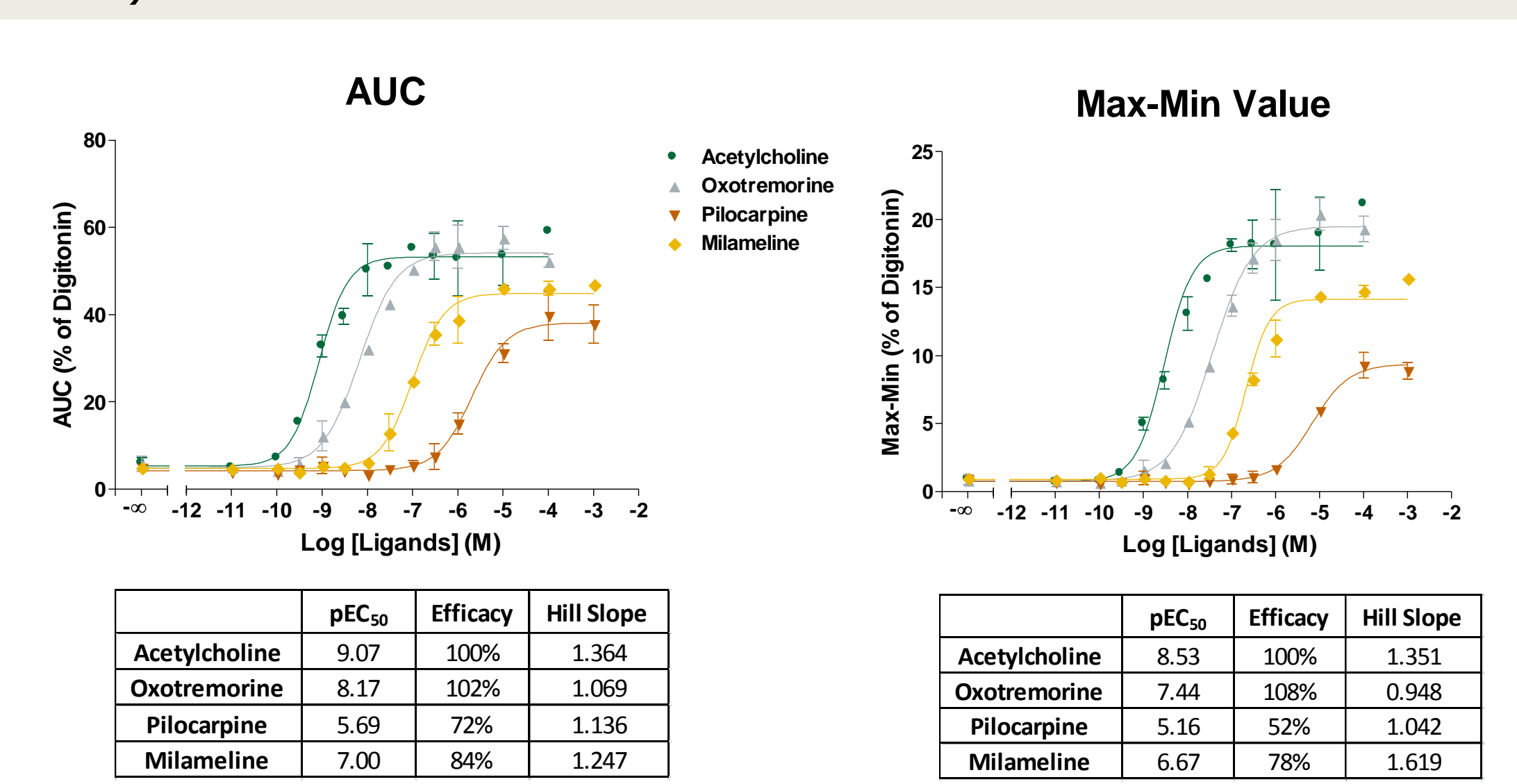
Agonists and antagonists rank order of potency was not modified by the method used for the analysis of data, as shown below for the muscarinic M_4 receptor agonist experiment (fig6), and for the Adenosine A_{2B} antagonist experiment. This was also true for the other receptors analyzed (4 in agonist mode, 3 in antagonist mode, data not shown).



Adenosine A_{2B} receptor Aequorin Antagonist Assay, same kinetic data treated with AUC and Max-Min method

6 Partial Agonists' Apparent Efficacy Varies with Data Analysis Method

Calcium flux assay with the Aequoscreen® method allows detecting partial agonist activity. We observed that the apparent efficacy of partial agonists is lower when using the "Max-Min" method compared to the "AUC" method, as shown below for the muscarinic M_4 receptor. The same observation was made with the other receptor analyzed (muscarinic M_1 receptor, data not shown).



Muscarinic M_4 receptor Aequorin experiment, same kinetic data treated with AUC and Max-Min method

8 Materials and Methods

Cells, Cell Culture and AequoScreen® assay:

The 100 stable aequorin cell lines used in this study were from the PerkinElmer catalog, and were each cultured and used in the aequorin assay according to their technical datasheet specifications (see www.PerkinElmer.com). Each receptor was tested in the aequorin assay in agonist mode with 3 to 8 replicates of each concentration of the reference agonist. Aequorin assays were performed using suspension cells, dispensed on the agonists present in the assay plates, and using either the LumiLux® (PerkinElmer) or the MicroLumat® LB96V (Berthold) readers. Raw kinetics data were exported in excel, and used to calculate the Area Under the Curve ("AUC" = sum of each individual timepoint), or to extract the maximal response, and the minimal response, and calculate their difference ("Max-Min", corresponding to the peak height). Fitting of data according to a sigmoidal dose response model (variable slope) was done using GraphPad Prism® software version 5.01, and parameters such as pEC_{50} , pIC_{50} , Hill slope and "TOP" of the curve generated by GraphPad® software were used to compare the "AUC" and "Max-Min" data analysis methods. GraphPad® software estimation of the TOP of the curve was better when using the AUC method, with 98% of curves with a TOP value being less than 20% bigger than the highest experimental point, vs 86% of curves for the Max-Min method fitting this criteria. **Fluorescent-dye based adherent assay methodology:** Fluorescent calcium assay was performed with Fluo-4 no wash (Invitrogen Cat n°F36206) following instructions from the supplier of the kit. Responses were measured on CellLux® instrument (PerkinElmer) (λ_{ex} . 485nm, λ_{em} . 535nm).

9 Conclusions

□ Calcium flux using AequoScreen® assays is a convenient and cost-effective method to perform HTS as well as pharmacological characterization of compounds.

□ The method used to transform the kinetic luminescence data into sigmoidal dose-response curves impacts the apparent pharmacological parameters that can be extracted from these curves, such as EC_{50} , IC_{50} and efficacy values. In addition, the experimental settings (like dispensing speed, dispensing height, geometry of the reader used,...) can also modify the kinetics (not shown) and hence the values of the parameters that can be extracted from them.

□ The method we recommend to analyze aequorin data is using the Area Under the Curve (AUC) of the luminescent signal, due to the better sensitivity (higher pEC_{50} values), the better assay quality (Z' value), and the better curve fitting when using this method.

□ As for any assay, the true meaning of parameters such as EC_{50} , IC_{50} and efficacy extracted from aequorin experiments results must be kept in mind when using them and when comparing them with results from other assays.