Screening for positive allosteric modulators of a GPCR with a three-addition Ca^{2+}-flux protocol

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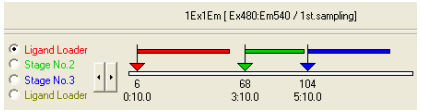
Introduction

We have established a Ca^{2+}-flux assay protocol to measure activation of an undisclosed GPCR by its natural ligand in a stably transfected cell line on FDSS6000 or FDSS7000* readers.

In this three-addition protocol, compounds are added to cells, followed by stimulation with two different concentrations of ligand, e.g. EC_{20} and EC_{80}. Thus, within the same experiment test compounds were screened for agonistic and antagonistic effects, but also for positive allosteric modulators (PAM) which act only in conjunction with endogenous ligand, and which would not be identified otherwise.

We show that the sequential addition of two different concentrations of ligand has no detrimental effects on the potency of reference antagonists.

In a HTS with 85,000 compounds, high quality data (Z'-factor and day-to-day reproducibility) were obtained. Follow-up experiments are currently under way.



* A FDSS7000 reader was courteously provided by Hamamatsu Photonics for evaluation purposes (www.hamamatsu.com).

Assay protocol

Ca^{2+}-flux assay

CHO cells stably transfected with an undisclosed GPCR were seeded onto black clear-bottom tissue culture-treated plates and incubated overnight at 37°C, 5% CO_2.

On the day of assay medium was removed and 50 µl of dye solution added, to final concentrations of 3 µM Fluo4-AM, 5 mM probenecid. Cells were incubated for 1 hr at 37°C, 5% CO_2 and equilibrated at RT for 30 min before assay in the FDSS6000 or FDSS7000. No further wash steps were required.

Three-addition protocol on FDSS6000 and FDSS7000

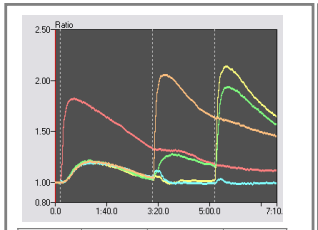
Our FDSS6000 has two separate dispensing heads. Head 1 was used for transfer of compounds and for the EC_{20} concentration of the ligand; Head 2 was used for transfer of the higher ligand concentration, avoiding problems with carryover. Essentially the same program was used on the FDSS7000.

1st addition	Head 1 Compound plate on loader Transfer 10 ul, collect RFU traces for 3 min
2nd addition	Head 1 Ligand plate on stage 2 Transfer 10 ul, collect RFU traces for 2 min
3rd addition	Head 2 Ligand plate on stage 3 Transfer 10 ul, collect RFU traces for 2 min

Results

Setup of three-addition protocol and validation with reference compounds

A three-addition Ca^{2+}-flux protocol was set up on FDSS6000 and FDSS7000. The assay protocol was validated with dose response experiments with three reference compounds; an example of dose response curves of a well-characterized compound measured on both devices is shown. Table 1 shows experimental IC_{50} values of three reference substances, compared to expected IC_{50} values.



Trace color	1st addition	2nd addition	3rd addition
	EC_{100}	-	-
	Buffer	EC_{100}	-
	Buffer	Buffer	EC_{100}
	Buffer	Buffer	Buffer
	Buffer	EC_{20}	EC_{80}

Figure 1. Ratio traces in three-addition protocol.

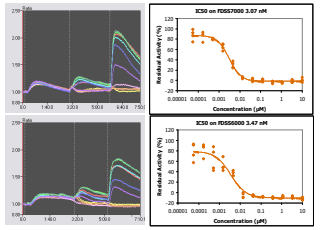


Figure 2. Titration of reference compound in three-addition protocol on FDSS7000 (top) and FDSS6000 (bottom), calculated dose-response curves and IC_{50} values. red ratio trace: 100% control; yellow ratio trace: buffer control.

	Experimental	Expected
Reference 1	3-5 nM	6 nM
Reference 2	25-40 nM	20 nM
Reference 3	1 nM	2 nM

Table 1. Experimental and expected IC_{50} values of three reference inhibitors.

The IC_{50} values of three reference compounds, calculated from data of the 3rd addition, were not affected if cells were stimulated twice, with EC_{20} and EC_{80} of the ligand. Comparable results were obtained with FDSS7000 and FDSS6000. Overall, RFU signals and S/B ratios were slightly higher in FDSS7000.

Results of HTS of >85,000 compounds

In the HTS >85,000 compounds on 214 assay plates were screened. Figure 3 shows results of a typical assay plate, heat maps of relative activity Ar% and examples of ratio traces of putative agonists, assessed after the 1st addition, positive allosteric modulators, assessed after the 2nd addition, and antagonists, assessed after the 3rd addition. Quality control and cut-off criteria are given in Table 2.

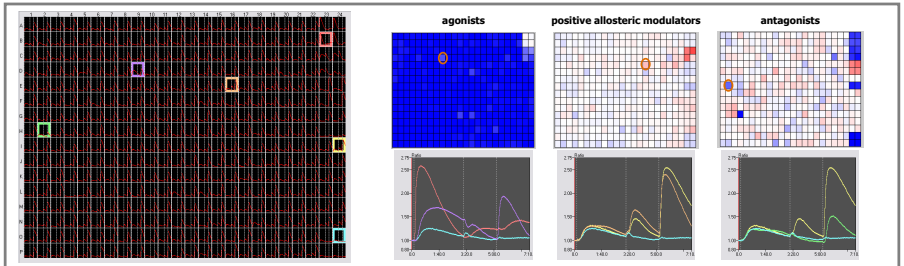


Figure 3. Examples of ratio traces on FDSS6000 and correlation to heat maps; an assay plate showing typical examples of agonists, positive allosteric modulators and antagonists is shown. Red: positive control 1st addition; yellow, positive control 3rd addition; blue: negative control.

Number of compounds screened	86'159		
Evaluation mode	**Agonists**	**Antagonists**	**PAM**
Mean Z'-factor	0.76	0.80	n.d.
Mean S/B ratio	2.02	2.15	n.d.
Agonist hit rate (Ar>35%)	0.25%		
Confirmation rate after verification	**32%**		
Antagonist hit rate (Ar<40%)		0.38%	
Confirmation rate after verification		**58%**	
PAM hit rate (Ar>120% re. sample mean)			1.31%

Table 2. Mean Z'-factor and S/B ratios, cut-off criteria for hits and confirmation rates.

Compounds fulfilling cut-off criteria were re-tested in duplicate:

- Primary agonist hits ➢ re-tested in three-addition protocol
- Primary antagonist hits ➢ re-tested without 2nd addition
- Primary positive allosteric modulatory hits:
  - ➢ re-tested in three addition protocol
  - ➢ re-tested in +/- EC_{20} of ligand on 2nd addition, to confirm ligand-specific enhancement of signal

Results of initial follow-up experiments with positive allosteric modulators

In an initial follow-up experiment compounds with confirmed modulatory activity in presence of EC_{20} of ligand were further tested. Effects of the compounds on cells stimulated with different concentrations of the ligand were assessed; Figure 4 shows an example where pretreatment with the compound resulted in a four-fold shift of the EC_{50} value of the ligand. Further experiments to characterize these compounds are currently under way.

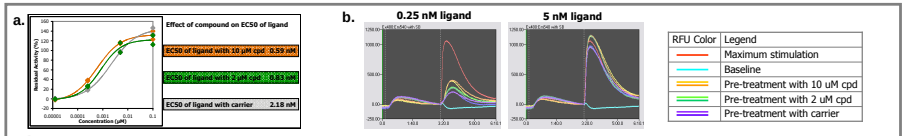


Figure 4. Effects of a putative positive allosteric modulator. **a.** Effects of pre-treatment with 10 µM (orange) or 2 µM compound (green) in 1st addition on different concentrations of ligand applied in the 2nd addition, compared to pre-treatment with carrier only (grey). **b.** RFU traces demonstrating the positive effects of the compound on treatment of cells with two different concentrations of ligand.

Conclusions

We have developed a three-addition Ca^{2+}-flux protocol on FDSS6000 and FDSS7000 which allows the screening for agonists, positive allosteric modulators and antagonists in the same experimental setup.

The protocol was validated with dose response experiments with three different reference inhibitors.

A HTS of >85'000 compounds was carried out, and resulted in a data set of high quality:

- Putative agonist hits were identified and activity confirmed with the same assay protocol; a confirmation rate of 32% was observed
- Putative antagonist hits were confirmed with a two-addition protocol, resulting in a confirmation rate of 58%
- Putative positive allosteric modulators were identified and are currently undergoing confirmation studies:
  - ➢ The evaluation procedure of the confirmation experiment was modified, thus no confirmation rate was calculated
  - ➢ PAM with confirmed activity were further tested in a two-addition protocol, in absence and presence of EC_{20} of ligand
  - ➢ Compounds with confirmed activity will be re-ordered in larger quantities for better characterization of their effects

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