Actelion Pharmaceutical
Allschwill

Hamamatsu 10th FDSS
User Meeting June 2014

Importance of calcium assay parameters in drug discovery

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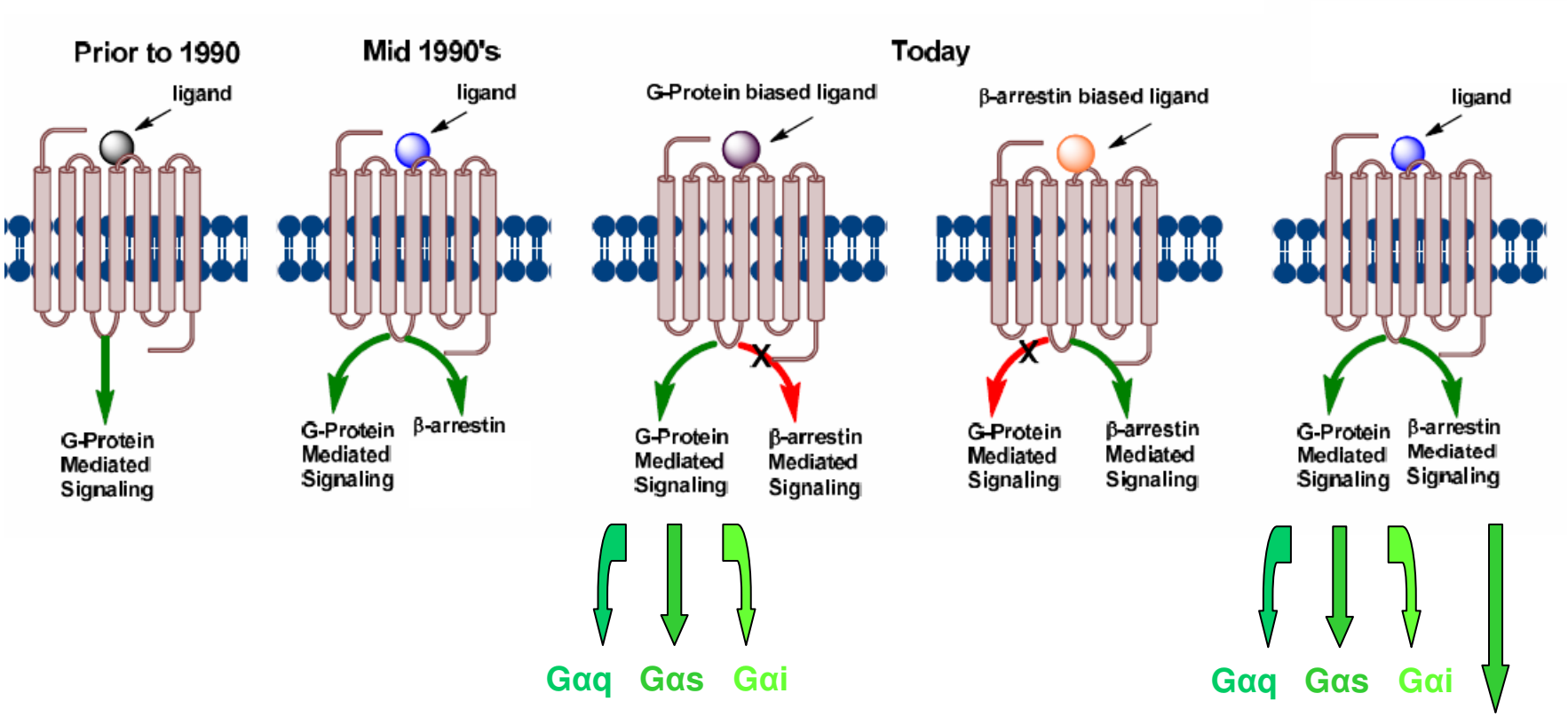
Success is the ability to go from one failure to another with no loss of enthusiasm (Sir Winston Churchill)

GPCRs signaling



Functional selectivity

Several ligand-specific receptor conformations

can be associated to biased functionnal signaling

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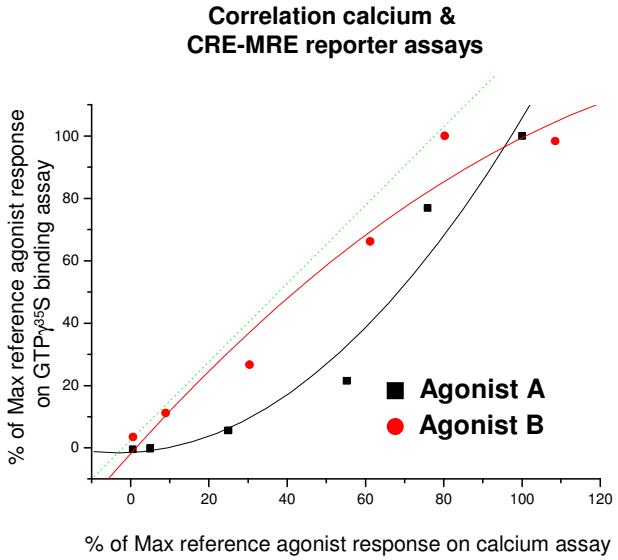
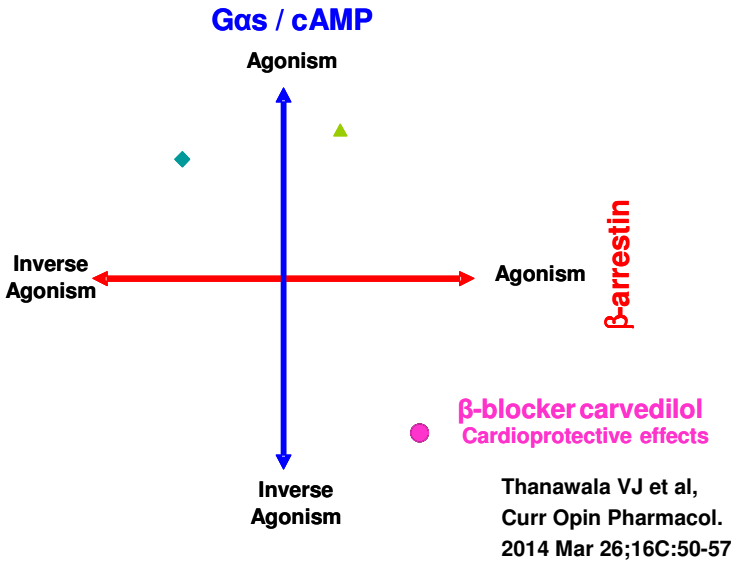
Precise affinity required for GPCR antagonism

- **Advance SAR analysis**
- **Studying drug specificity**
- **Accurate affinity values for pre-development compounds**
- **Identification of biased signaling**



Agonism



Thanawala VJ et al,
Curr Opin Pharmacol.
2014 Mar 26;16C:50-57

Antagonism

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Calcium mobilization assays at Bioprojet:

→ **HTS (Identify and classify hits)**

→ **Evaluate agonism efficacy and affinity**

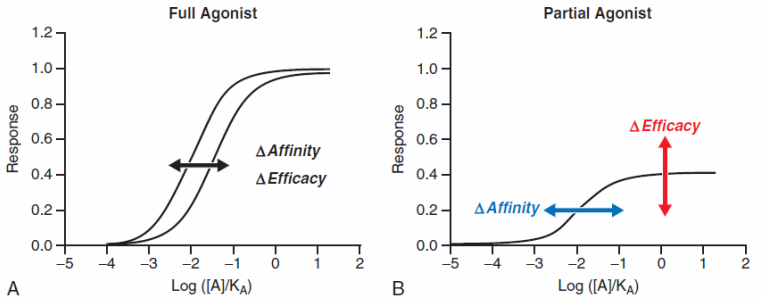

FIGURE 11.7 Sensitivity of various descriptive parameters for concentration-response curves to drug-receptor parameters. (A) The location parameter (potency) of curves for full agonists depends on both affinity and efficacy. (B) For partial agonists, the location parameter (EC_{50}, potency) is solely dependent upon affinity while the maximal response is solely dependent upon efficacy.

→ **Evaluate type of antagonism (Schild regression analysis)**

Type of Antagonism	pA_2 =	Correction Factor	Curve Pattern(s)
Competitive Surmountable	pK_B	None but Schild regression must be linear with unit slope	
Hemi-Equilibria	$pK_B + \psi Log(1+2[A]/K_A)$ $\psi << 1$	Very slight overestimation to no correction	
Orthosteric Insurmountable	$pK_B + Log(1+2[A]/K_A)$	Slight overestimation (maximal error ≈2)	
Allosteric Insurmountable	$pK_B + Log(1+2\alpha[A]/K_A)$	Very slight overestimation (for modulators with $\alpha < 1$)	

Fig. 7. Summary of the relationship between the pA_2 for various mechanisms of antagonism and the pK_B.

→ **Identification of biased ligands**

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GPCR antagonism and Calcium assay in drug discovery

Need to obtain precise affinity by Kb determination in Calcium assays

Kb is applicable at equilibrium conditions that are not encountered with functional calcium assays

(incubation exceeds 4 times the dissociation t 1/2 of ligand/receptor)

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Use of the pA2 as a universal determinant of antagonist potency

$$pA2 = pKb + \log (1 + 2 [A] / Ka)$$

At low [agonist] occupancy ⟶ $[A] << Ka$

$pA2 \sim pKb + \log (1)$ ⟶ **pA2 tend towards the pKb**

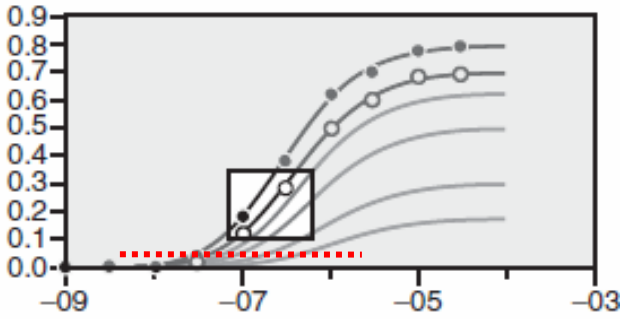
Calculation of pA2 at low agonist responses

→ **Overcome the potential bias associated with non equilibrium conditions**

→ **Estimate insurmountable antagonists affinity**

Concentration response curve dextral displacement

Max response reduction



Non equilibrium

- Steven J Charlton and Georges Vauquelin, 2010, British J Pharmacol 161:1250–1265
- Terry Kenakin, 2009, A pharmacology Primer: Theory, Application and Methods, Chapter 11, Academic Press
- Terry Kenakin et al, 2006, JPET 319:710–723
- Arthur Christopoulos et al, 1999, Euro J Pharmacol, 382:217–227

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pA2 = - log [M] of antagonist producing a 2 fold shift of the agonist concentration response curve

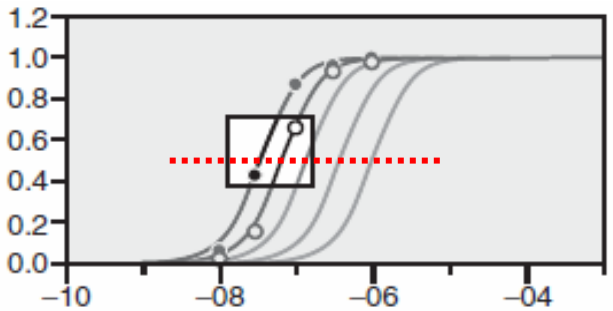
Use of Dose Ratio (DR) values as surrogate parameter for calculation of pA2

pA2 = log (DR – 1) – log B

At DR = 2 → **pA2 = – log B**

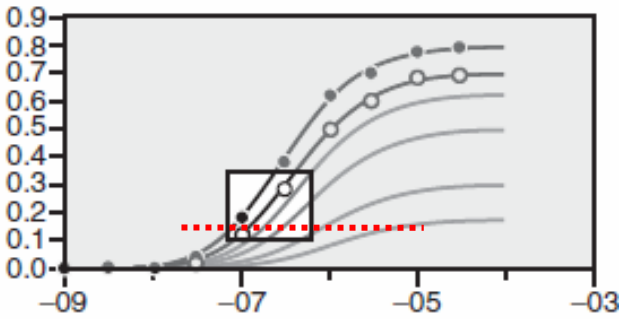
Competitive surmountable
Antagonism at equilibrium

DR at EC50



Non competitive (Insurmountable)
Antagonism at Hemi-equilibrium

DR at low agonist response



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Calcium assay parameters and GPCRs-ligand accessibility

1. Adherent vs suspension cells

2. Receptor functionality at the cell membrane

3. Ligand diffusion

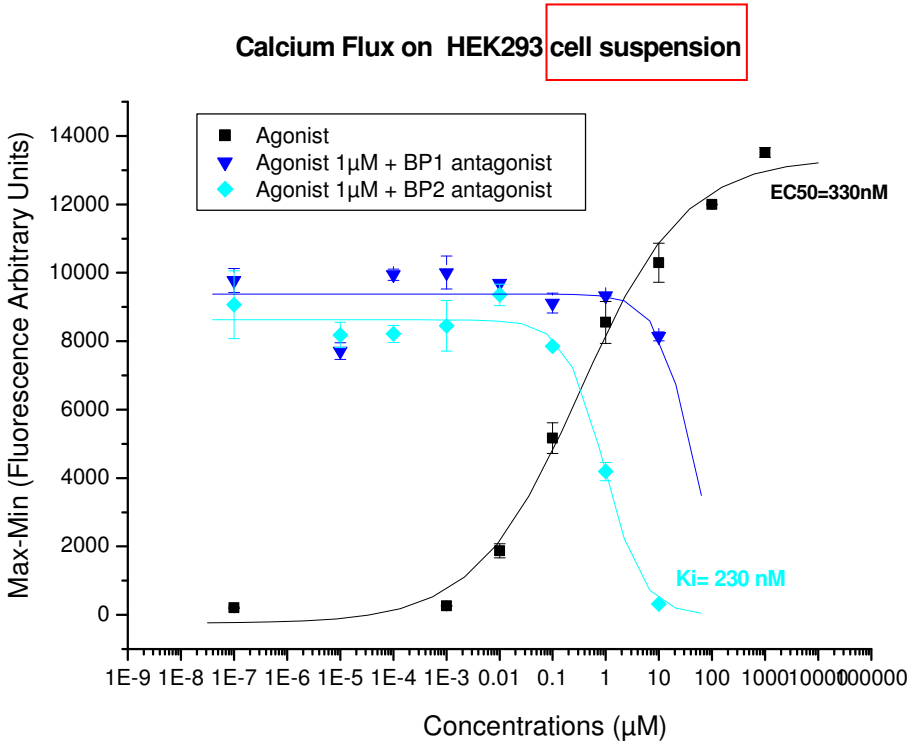
GPCRs and ligand accessibility

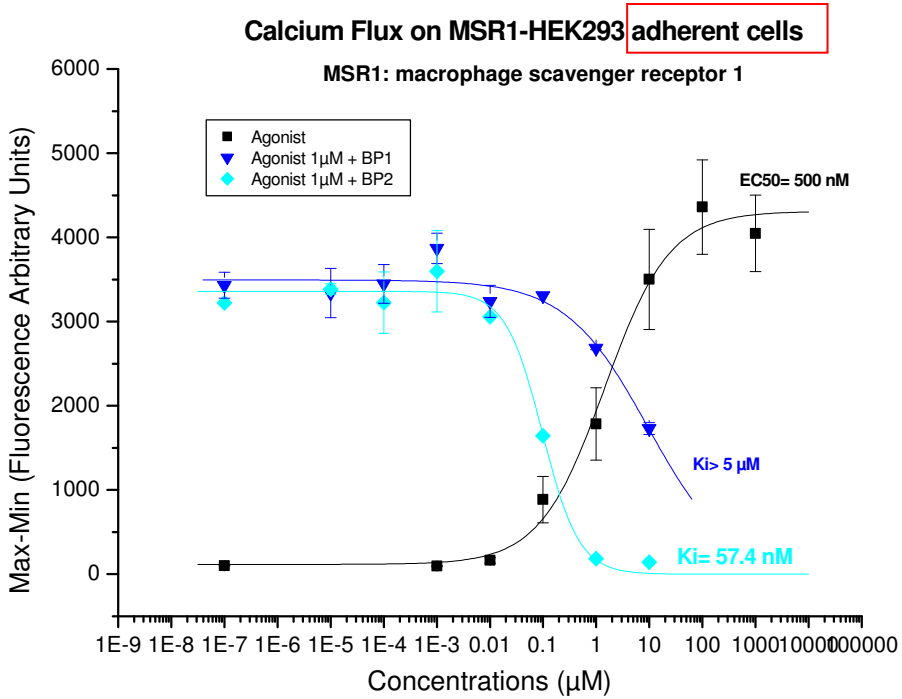
1. **Adherent vs suspension cells**

2. **Receptor functionality at the cell membrane**

3. **Ligand diffusion**

Settings: 10µl/sec, height 9.6 mm, sensitivity 200ms, gain 1





	Adherent Ki (nM)	Suspension Ki (nM)
BP2 antagonist	57 nM	230 nM
BP1 antagonist	> 5 µM	Inactive

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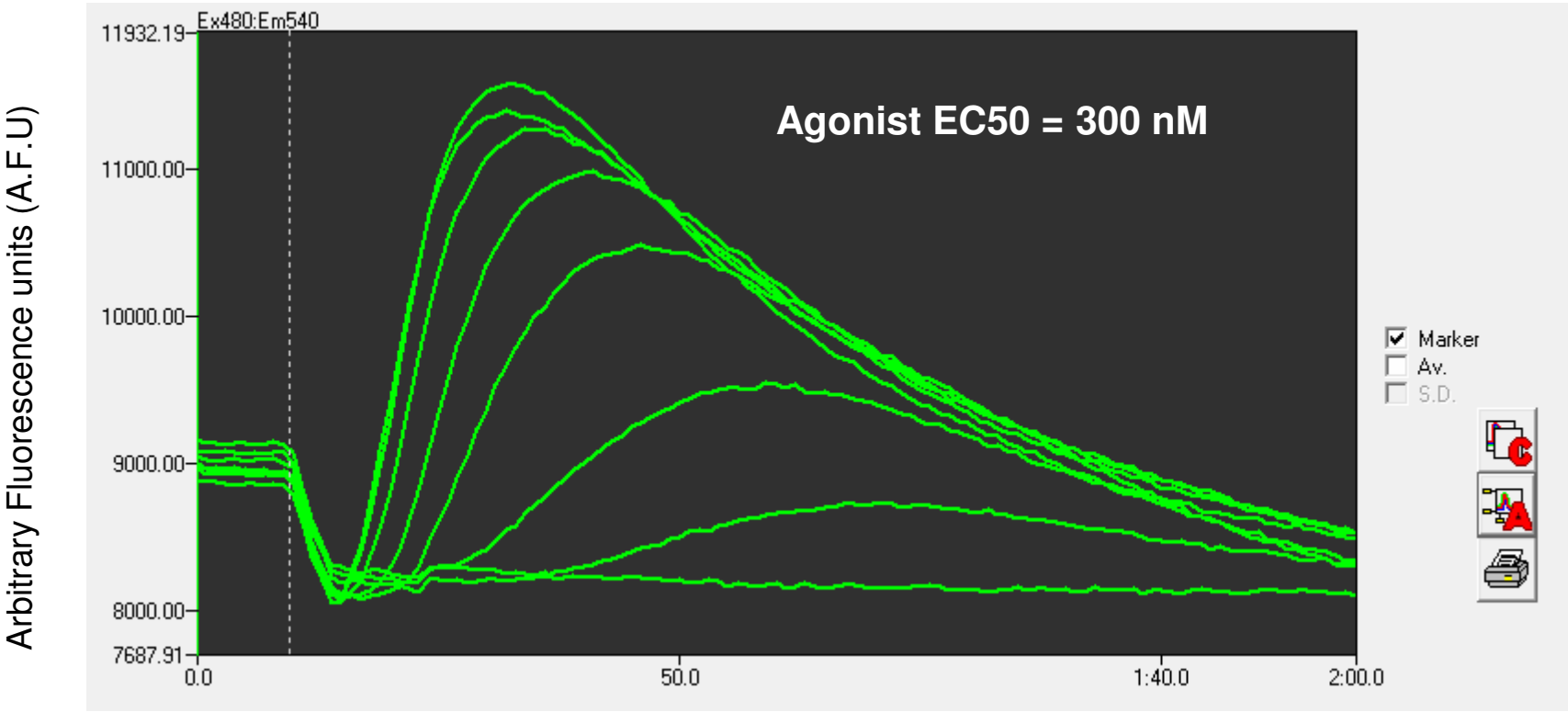
GPCRs and ligand accessibility

1. Adherent vs suspension cells

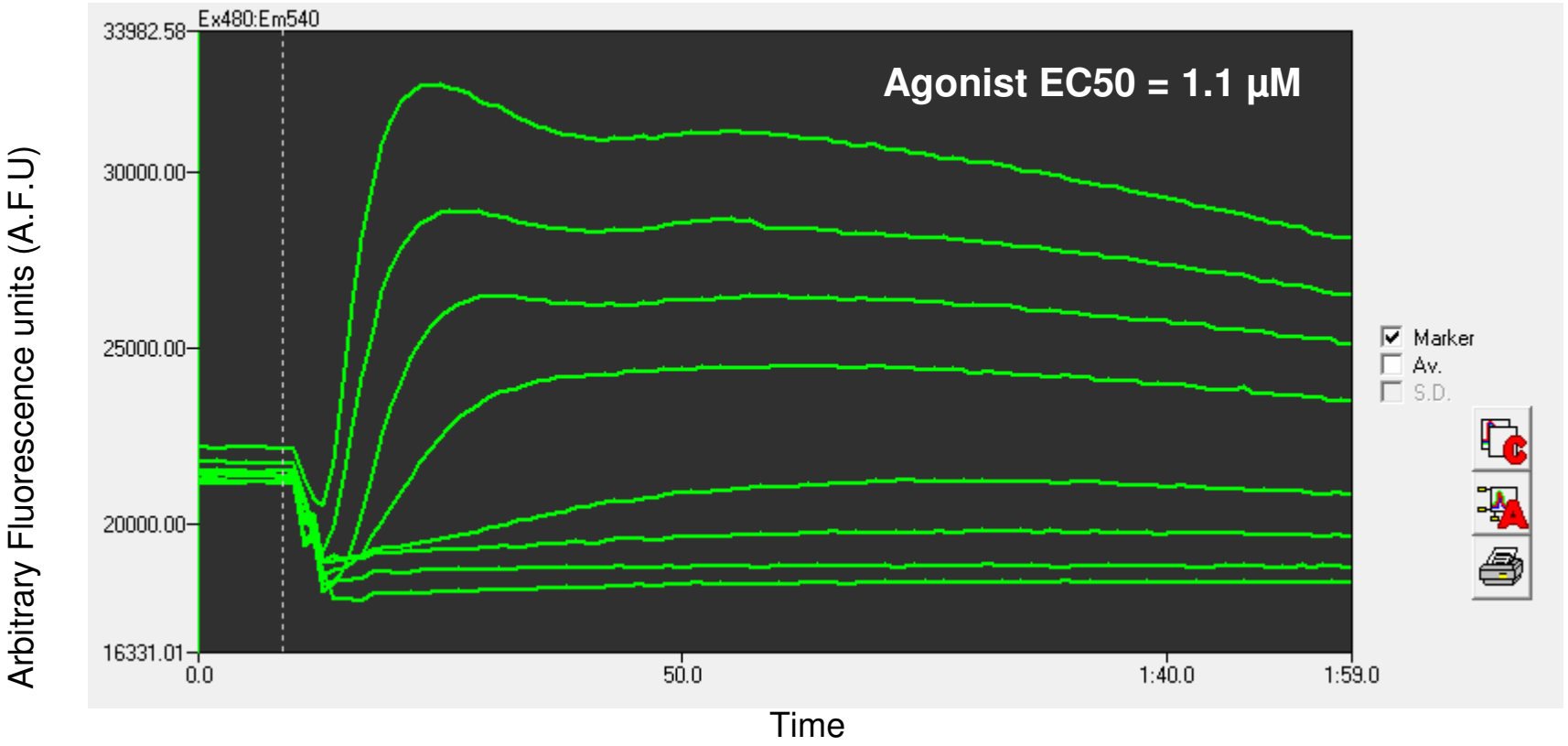
2. Receptor functionality at the cell membrane

3. Ligand diffusion

Calcium assay on recombinant-GPCR1 expressing HEK293 cells :



Calcium assay on native-GPCR1 in HUVEC cells :



Involvement of receptor reserve, agonist-induced structural modifications …. ?

Importance of GPCR expressing cells when looking at the calcium response

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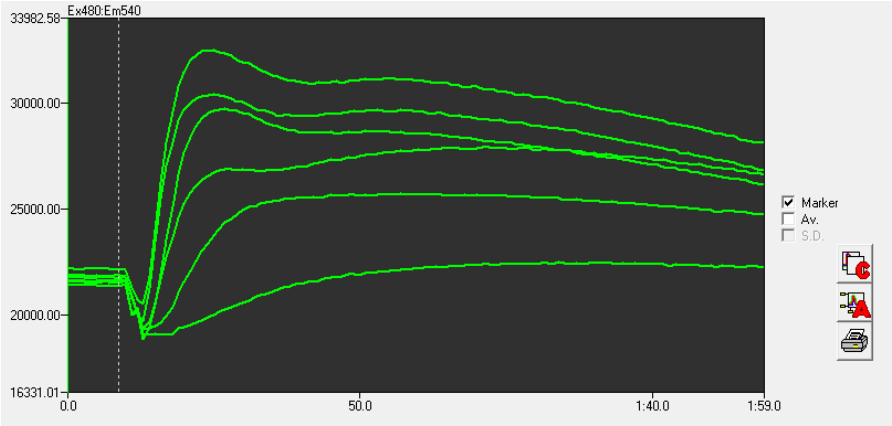
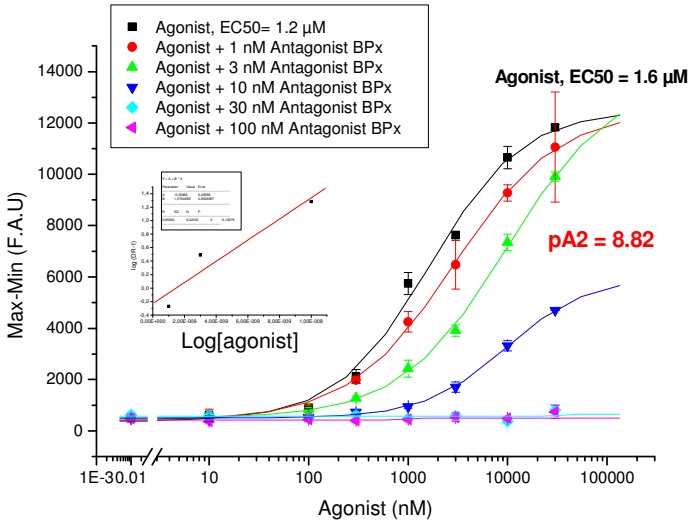
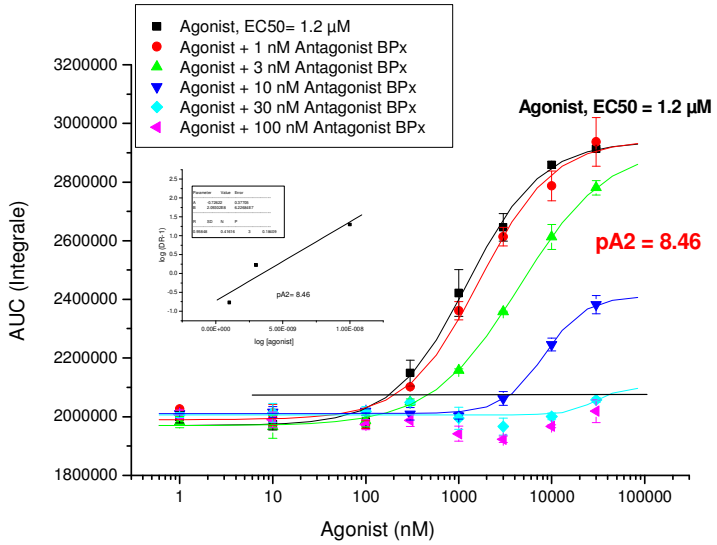
Calcium assay on native-GPCR1 expressing cells :

Evaluation of BPx antagonist (from 1nM to 100 nM) against 3µM reference agonist







No major difference observed when calculating pA2 using Max-Min or A.U.C data

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GPCRs and ligand accessibility

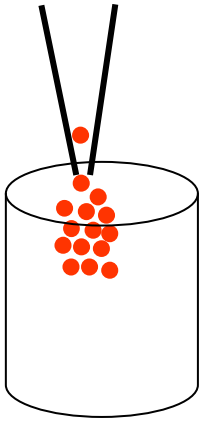
1. Adherent vs suspension cells

2. Receptor functionality at the cell membrane

3. Ligand diffusion

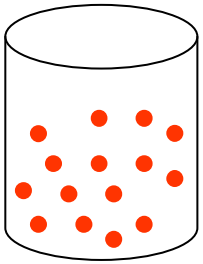
Calcium assay:

- Rapid and transient signaling system under non equilibrium condition
- Influenced by the diffusion characteristics of the injected agonist



Calcium assay:

- **Rapid and transient signaling system under non equilibrium condition**
- **Influenced by the diffusion characteristics of the injected agonist**



Diffusion

Movement of a fluid from higher concentration to lower concentration

The particles will mix until they are evenly distributed

This phenomenon of particles distribution is governed by the first and second laws of Fick

The diffusion phenomenom for the agonist may be of importance regarding :

- Depth and rate of agonist injection
- Nature and size of considered agonists (aminergic, lipidic, peptidic … ligands)
- Viscosity of the assay buffer (basic methodology vs NW kits)
- Volume and surface area of the assay well 96 well plate (full or ½ size wells)

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The diffusion phenomenom for the agonist may be of importance regarding :

- **Depth and rate of agonist injection (small molecule ligand)**

For antagonism charaterization

↓

Agonist injection: 10 µl / sec at 9.6 mm height

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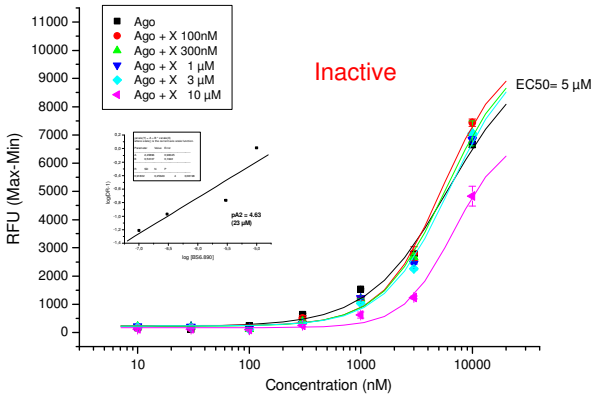
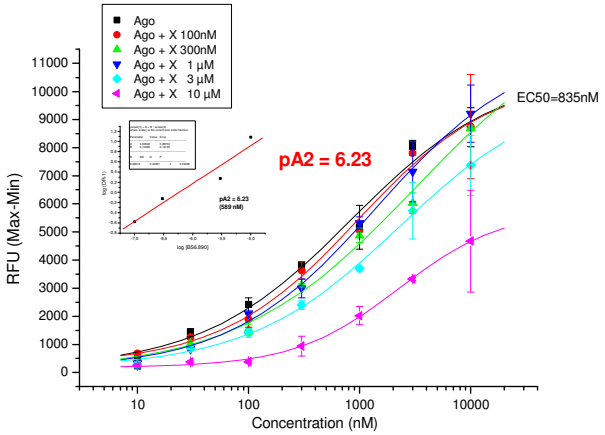
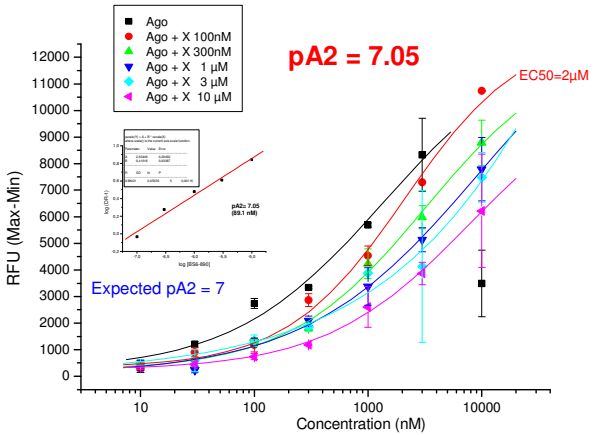
Compound BPx antagonism using CHO expressing recombinant hu-GPCR



100 µl height



180 µl height



240 µl height

Expected pA2 = 7

(FlexStation)

Agonist injection height (related volume)	FDSS µCell Determined pA2
4 mm (100 µl)	Inactive
7.2 mm (100 µl	6.23
9.6 mm (240 µl)	7.05

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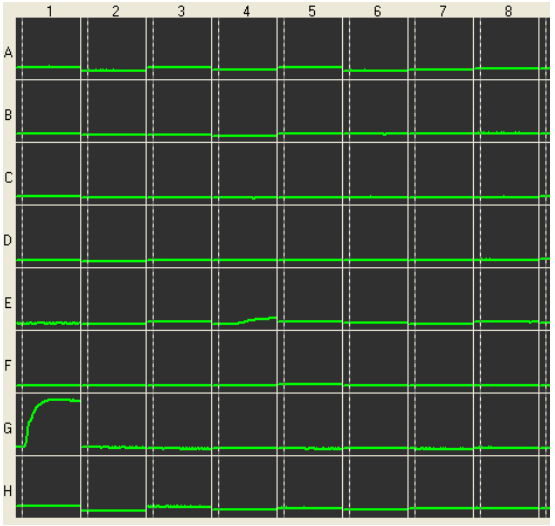
The diffusion phenomenom for the agonist may be of importance regarding :

- Depth and rate of agonist injection
- Nature and size of considered agonists (aminergic, lipidic, peptidic ligands)
- Viscosity of the assay buffer (basic methodology vs NW kits)
- Volume and surface area of the assay well 96 well plate (full or ½ size wells)

CHO-GPCR cells in 96 well plate

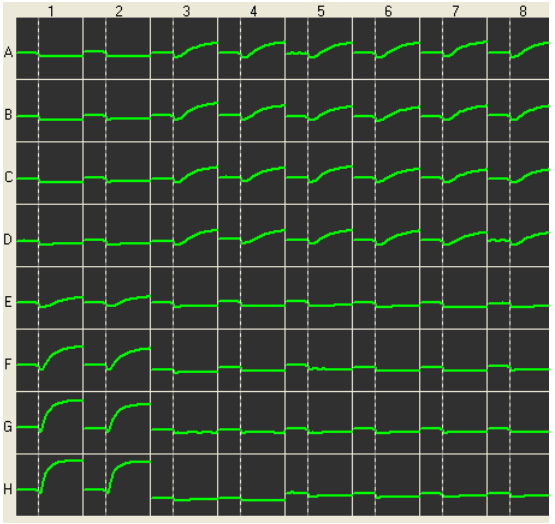
Antagonism study using
large peptidic endogenous agonist



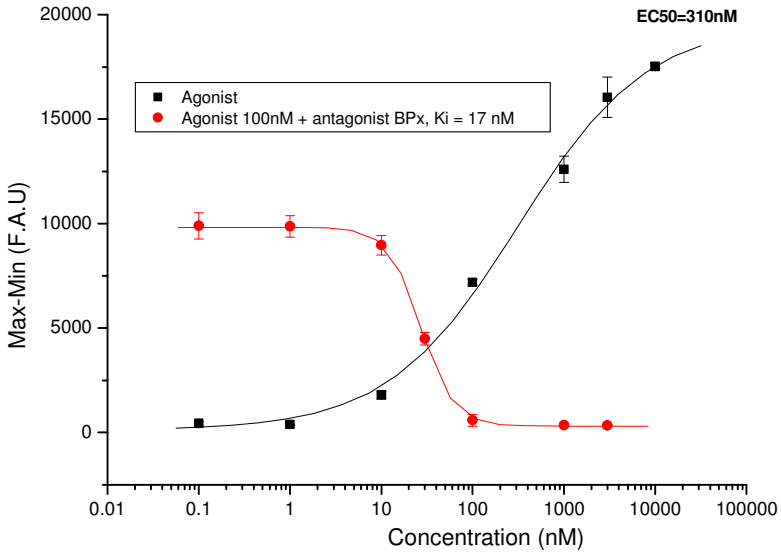
Settings:
10µl/sec, height 3 mm
sensitivity 200ms, gain 2

→ **No signal**

Determined Ki for antagonist and EC50 for agonist far from expected
→ ~ 1 nM and 30 nM, respectively



Settings:
80µl/sec, height 3 mm
sensitivity 200ms, gain 2



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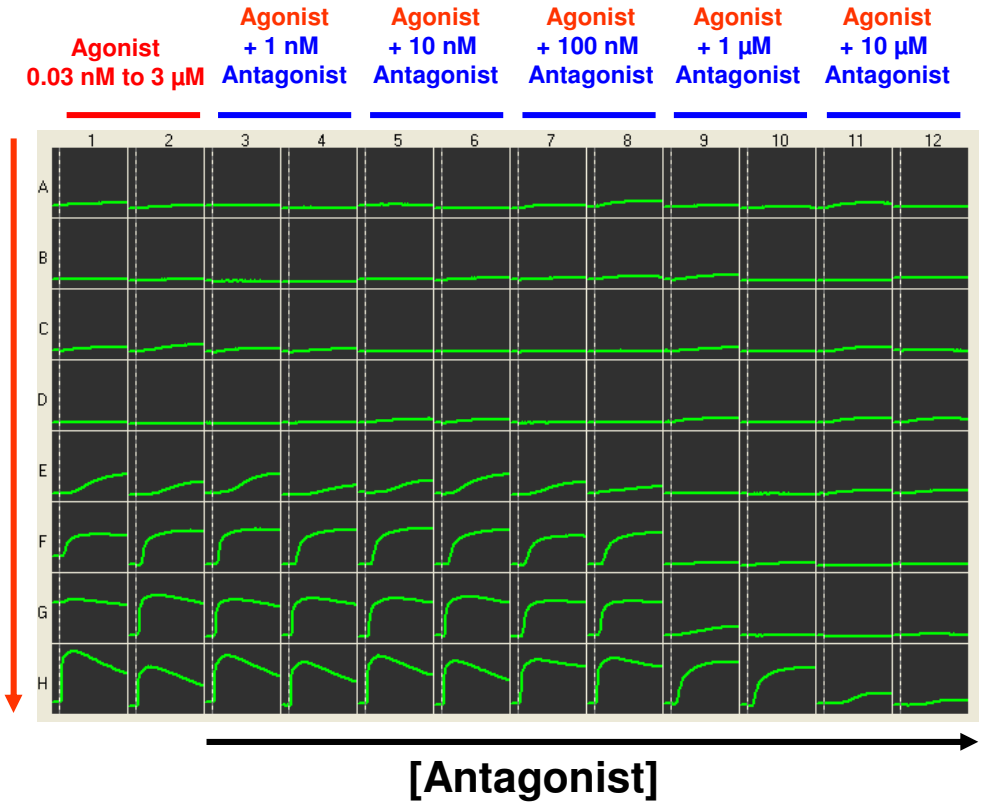
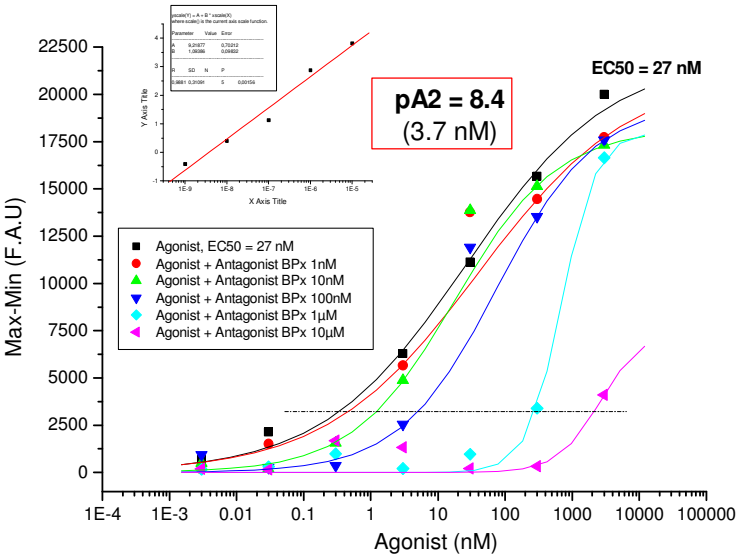
Antagonism study on CHO-GPCR cells with large peptidic agonist

Settings: 200µl/sec, height 3 mm, sensitivity 200ms, gain 2



Schild regression analysis



Speed injection	Agonist EC50 (nM)	Antagonist Ki (nM)
10 µl / sec	Inactive	Inactive
80 µl / sec	310	17
200 µl / sec	27	3.7

With large peptidic ligand, fast agonist injection is required to study antagonism

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The diffusion phenomenom for the agonist may be of importance regarding :

- Depth and rate of agonist injection
- Nature and size of considered agonists (aminergic, lipidic, peptidic ligands)
- Viscosity of the assay buffer (basic methodology vs NW kits)
- Volume and surface area of the assay well 96 well plate (full or ½ size wells)

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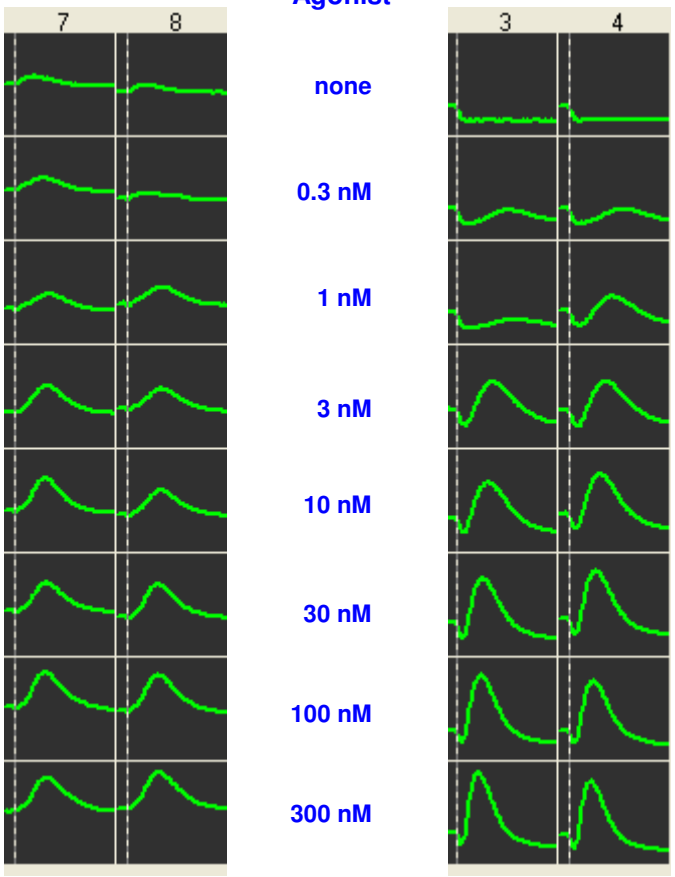
HEK293 cell in 96 well plate

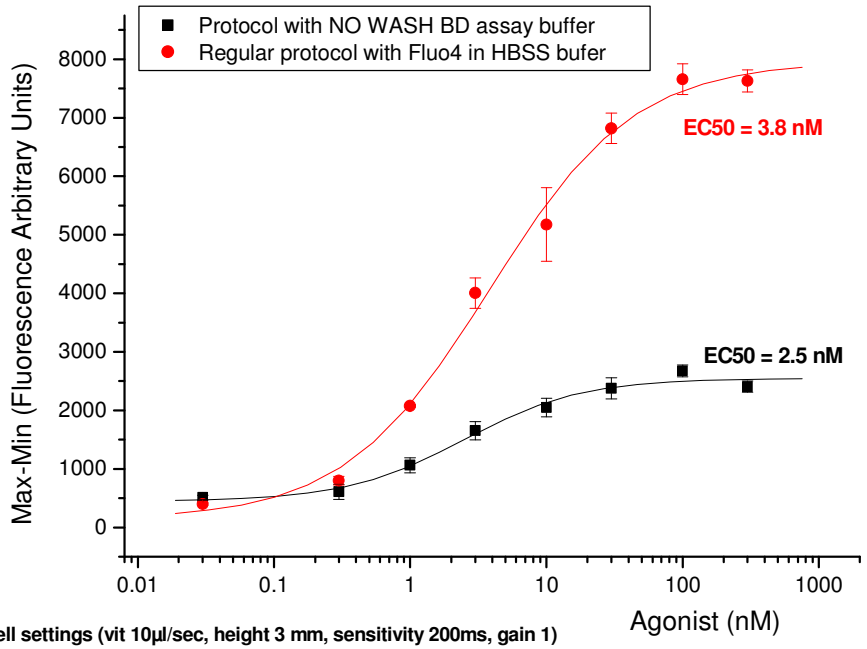
Settings: 10µl/sec, height 3 mm, sensitivity 200ms, gain 1

No Wash BD kit assay buffer

Regular protocol Fluo4 in HBSS assay buffer



HEK293 cell suspension in 96 well plate



µCell settings (vit 10µl/sec, height 3 mm, sensitivity 200ms, gain 1)

Working window is too narrow to study antagonism at 10 µl/sec agonist injection

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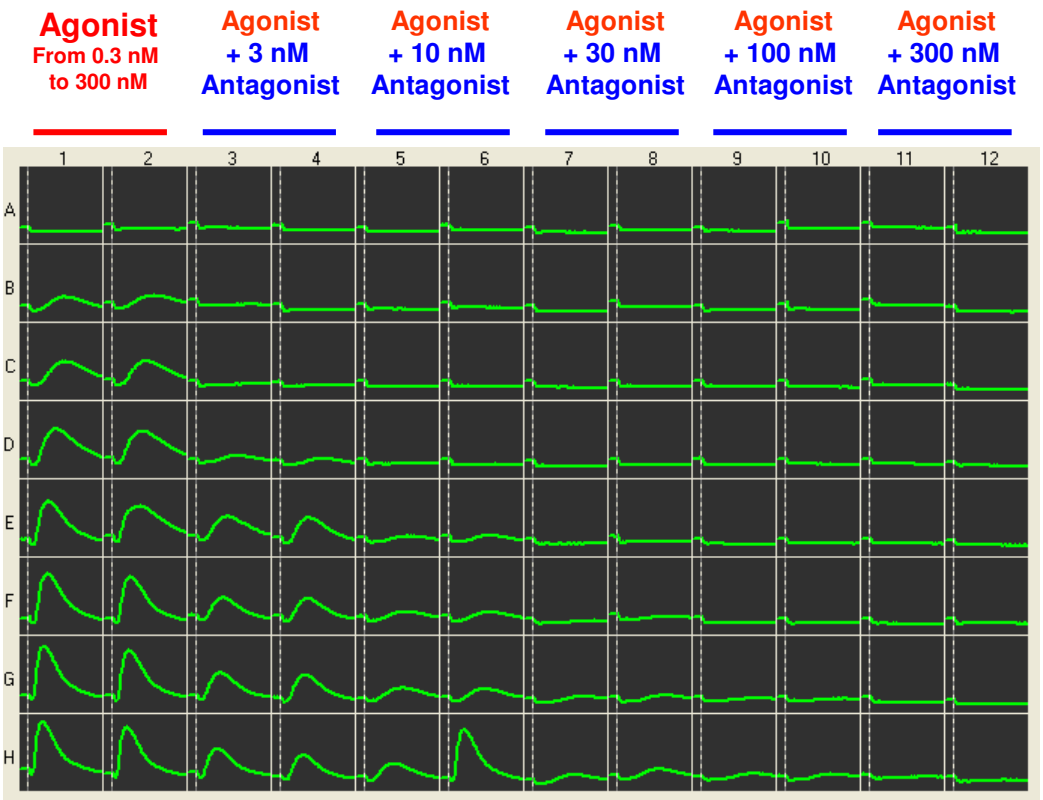
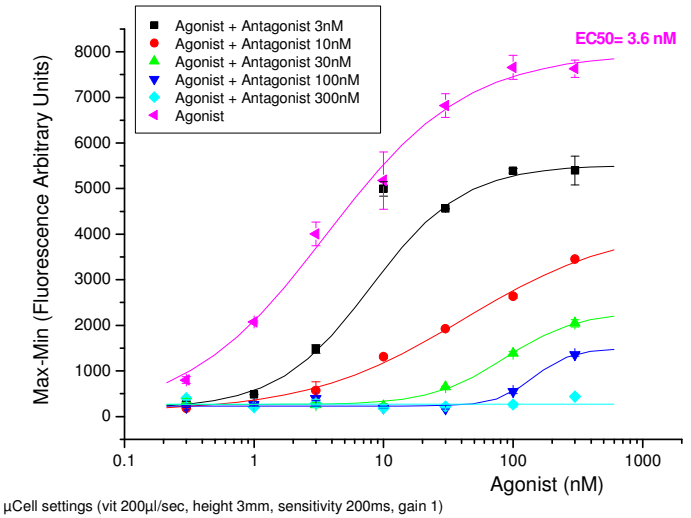
HEK293 cell in 96 well plate

Antagonism study using No Wash BD kit assay buffer

Settings: 200µl/sec, height 3 mm, sensitivity 200ms, gain 1



Schild regression analysis



µCell settings (vit 200µl/sec, height 3mm, sensitivity 200ms, gain 1)

In No Wash buffer, At 200 µl/sec agonist injection,
the Kb (pA2) can be determined for an antagonism

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The diffusion phenomenom for the agonist may be of importance regarding :

- Depth and rate of agonist injection
- Nature and size of considered agonists (aminergic, lipidic, peptidic ligands)
- Viscosity of the assay buffer (basic methodology vs NW kits)
- Volume and surface area of the assay well 96 well plate (full or ½ size wells)

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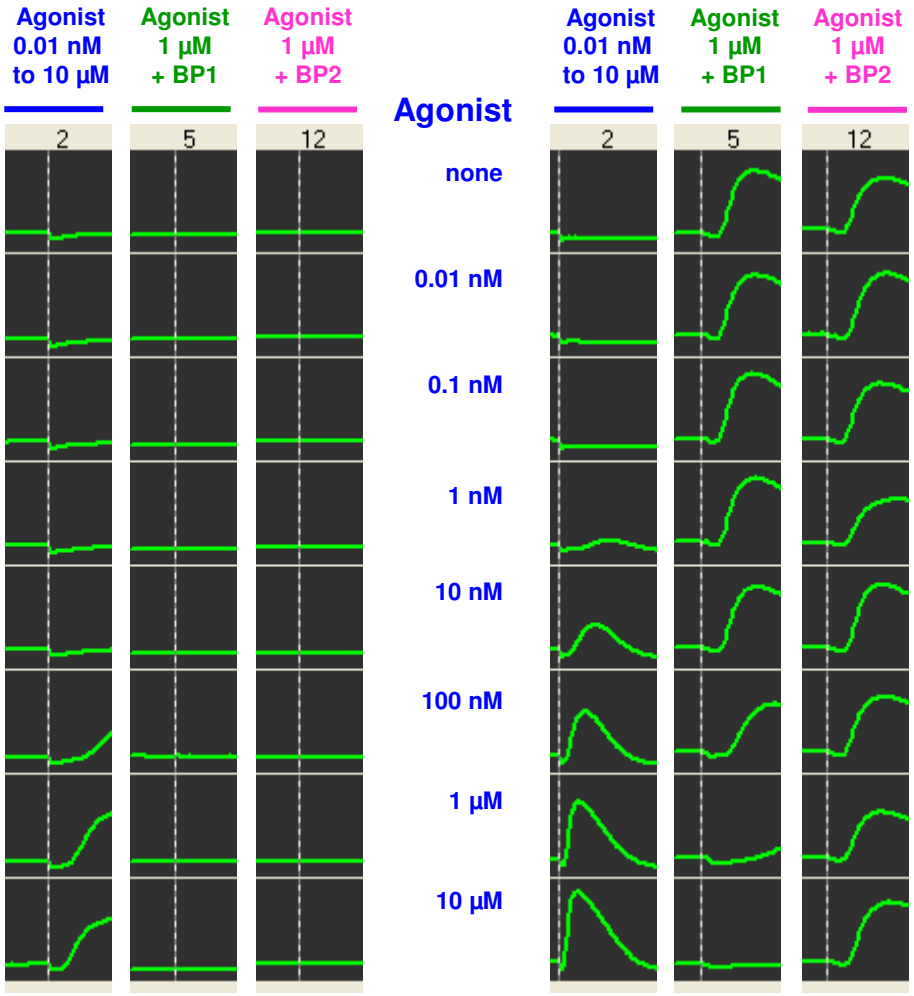
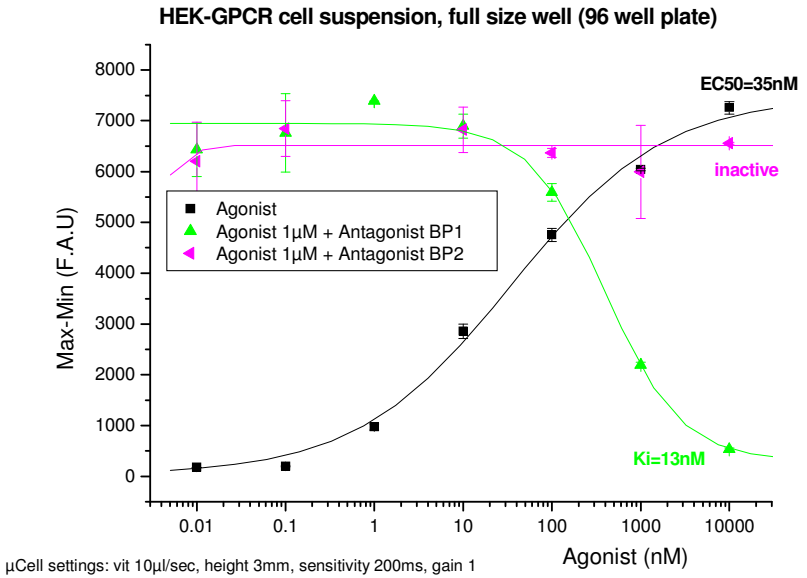
HEK-GPCR cell suspension in 96 well plate

Settings: 10µl/sec, height 9.6 mm, sensitivity 200ms, gain 1

1/2 size well

Full size well





µCell settings: vit 10µl/sec, height 3mm, sensitivity 200ms, gain 1

Settings for full size well are not compatible with ½ size well

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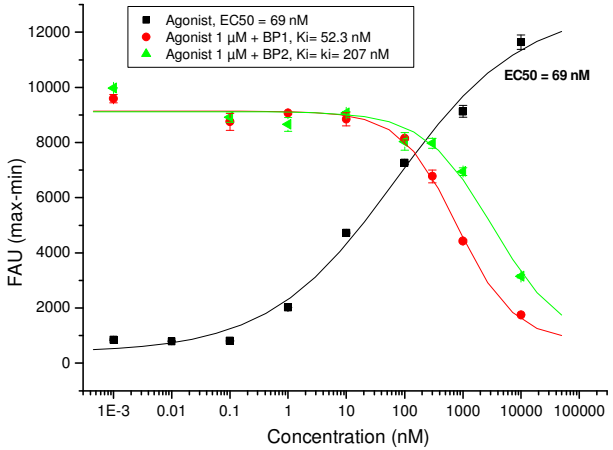
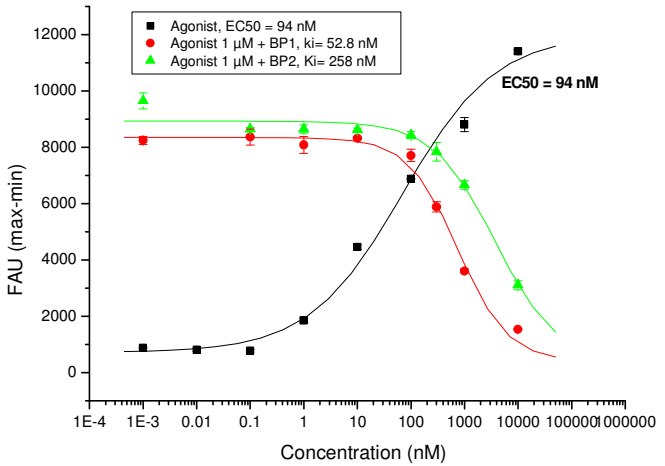
Antagonism study using ½ size well

Settings: 200 µl/sec, height 3 mm
sensitivity 200ms, gain 1



	96 Well plate	
	1/2 size	Full size
Agonist EC50 (nM)	69	94
Antagonist BP1 Ki (nM)	52.3	52.8
Antagonist BP2 Ki (nM)	207	258

Settings: 10 µl/sec, height 3 mm
sensitivity 200ms, gain 1



There is no differences when Ki is calculated from experiments done in ½ or full size well, at 3mm height but at different rate agonist injection

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SUMMARY

- Use of the pA2 for antagonist potency to overcome the potential bias associated with non equilibrium conditions

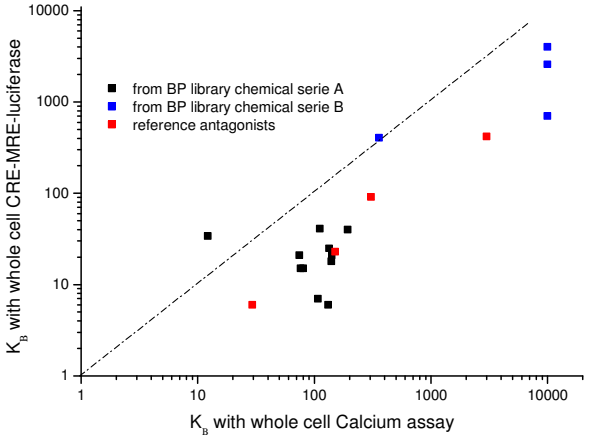
- Precise and defined agonist parameters needed for any given GPCR when implementing calcium assay

  - Receptor functionality at cell membrane
  - GPCR expressing cells
  - Ligand diffusion

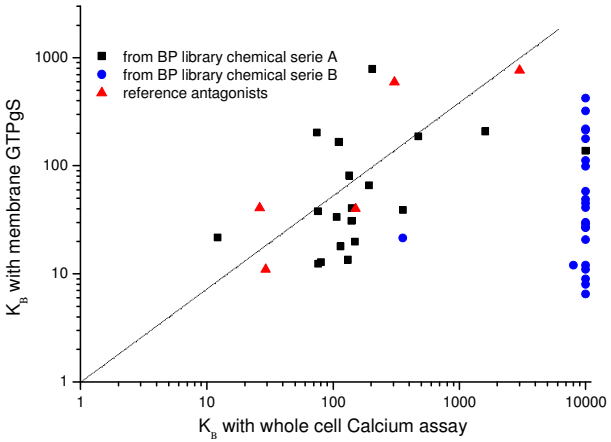
- Other important parameters to consider

  - Binding kinetics
  - Receptor trafficking
  - Receptor homo/hetero oligomerization

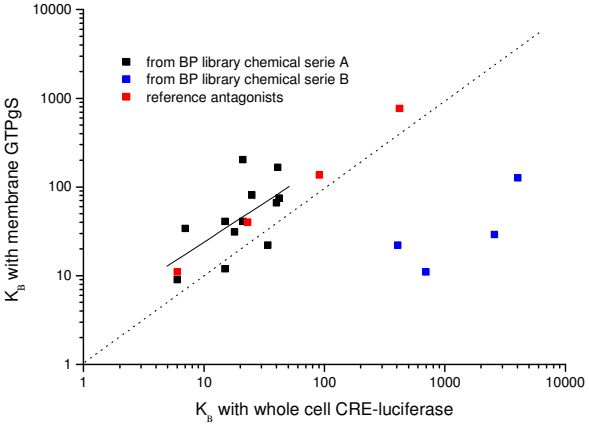
Correlation calcium & CRE-MRE reporter assays

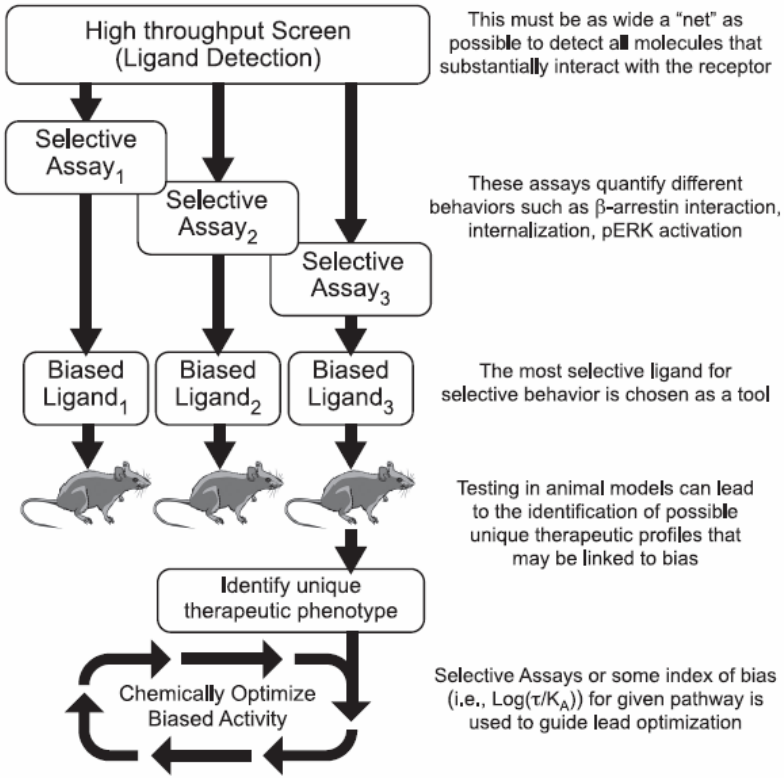


Correlation calcium & GTPγ^{35}S binding assays



Correlation CRE-MRE reporter & GTPγ^{35}S binding assays





***In fine*, we have to keep in mind that what really matters is *in vivo* therapeutic efficacy …**

Terry Kenekin, JPET 336:296–302, 2011

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