



Photina™: an improved Ca²⁺-sensitive photoprotein

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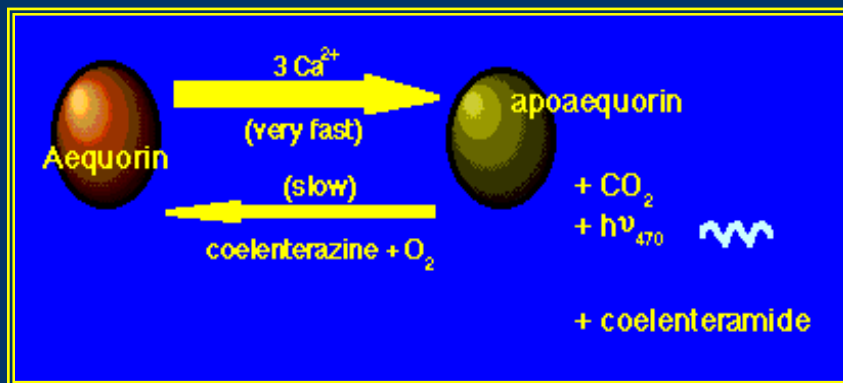
Topics of the Presentation

- Photoproteins
 - Characteristics
 - Applications
 - Biology
 - Structure
- Photina™
 - Generation
 - Characteristics
 - Applications



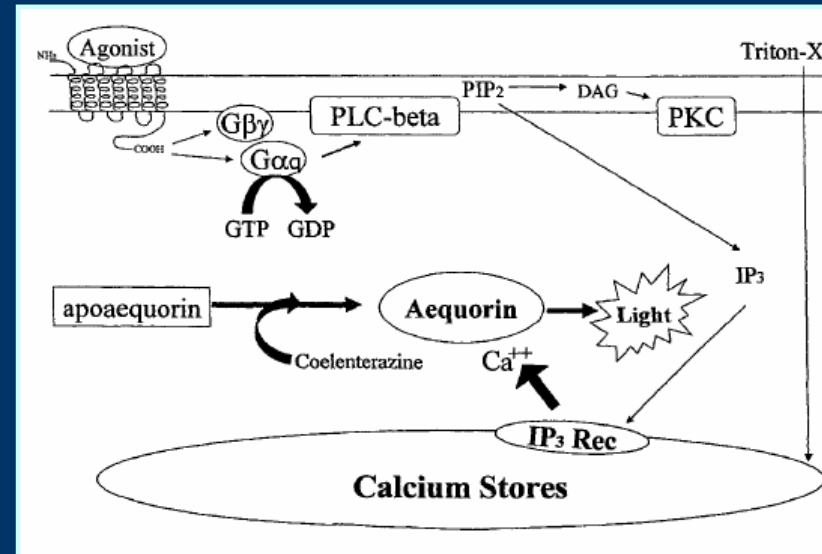
Bioluminescence

- Bioluminescence is light produced by a chemical reaction within an organism.
- **Photoproteins** are made up of both luciferin and luciferase, along with some other cofactor such as oxygen.
- Each photoprotein has **three calcium binding sites**.
- When calcium binds, the photoprotein becomes an apoprotein and **light and oxyluciferin are released**.
- The reaction is characterized by immediate **photon release** (flash luminescence) upon calcium binding to the coelenterazine-photoprotein complex.



Application of Calcium-sensitive Photoproteins

- Calcium-sensitive photoproteins are important tools for analysing calcium-mediated signal transduction processes in mammalian cells.
- As reporter system they are extremely useful for studying receptor-ligand interactions or ion channels when calcium mobilisation is involved.
- Extremely **sensitive method**.
- It reflects very well the natural signal transduction pathway.
- Signal transduction **within seconds**, eliminating therefore toxic compound effects.
- It requires very good assay and equipment set-up.



Assays for HTS based on Intracellular Calcium Increase

- GPCRs coupled to Gq
- GPCRs artificially coupled to Gq
- Ligand gated Ca^{2+} ion channels
- TRP channels
- Na^+ / Ca^{2+} exchangers



Flash Luminescence: Axxam's Experience and Know-how

Use of flash luminescence

- Several years of experience in setting up cell based assays using flash luminescence
- CCD camera system for measuring flash luminescence

Collaborations with Marine Institutes

- Biological material, consultancy

Cloning of new photoproteins

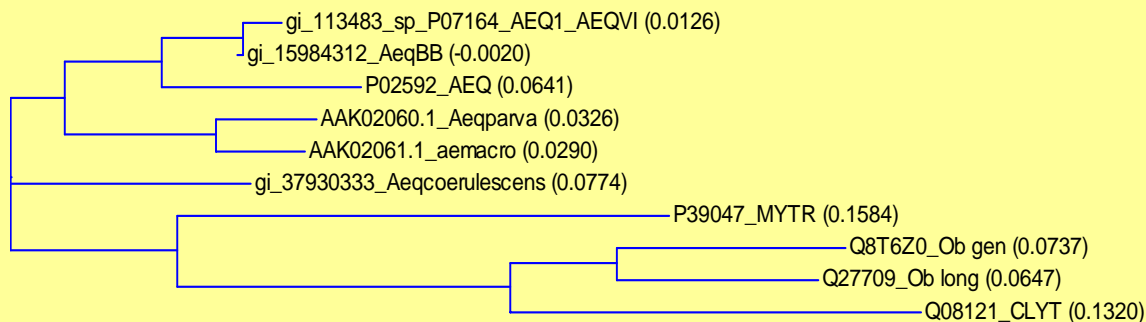
- Characterisation and patent protection

Modification of known photoproteins

- Improvement of quantum release
- Higher sensitivity to calcium
- Targeting to different cell compartments
- Increased protein stability



Sequence Comparison of known Photoproteins



	AEQ	AEQmacro	AEQparva	Ob long	Ob gen	MYTR	CLYT
AEQ	100	78	79	64	61	62	58
Aeqmacro		100	94	64	65	68	61
Aeqparva			100	65	63	67	60
Ob long				100	86	63	75
Ob gen					100	63	75
MYTR						100	59
CLYT							100



Photina™

Photina™ is a novel calcium-sensitive photoprotein, which:

- is derived from the consensus sequence of the known photoproteins,
- has a humanized codon usage for all codons,
- has a reduced number of cysteine residues,
- has an overall sequence similarity at the amino acid level of 70% to Aequorin.



Characteristics and Characterisation of Photina™



“In vitro”

- Light production

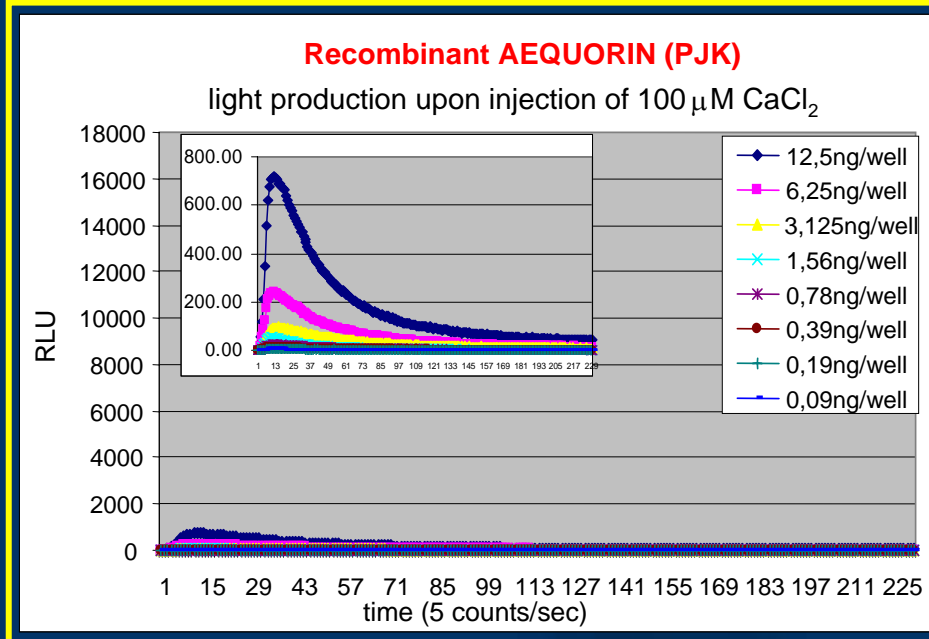
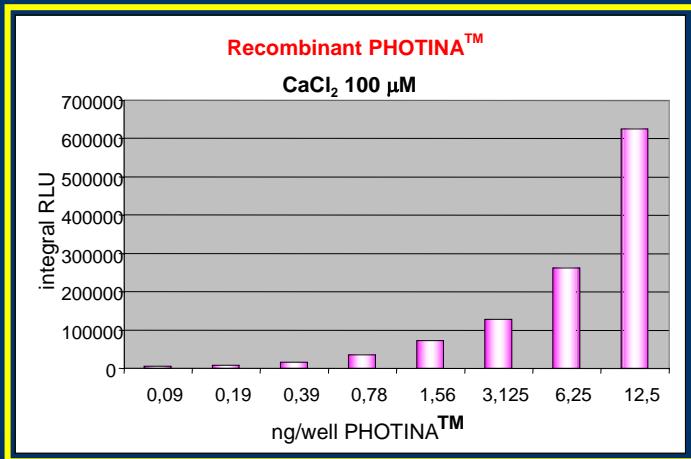
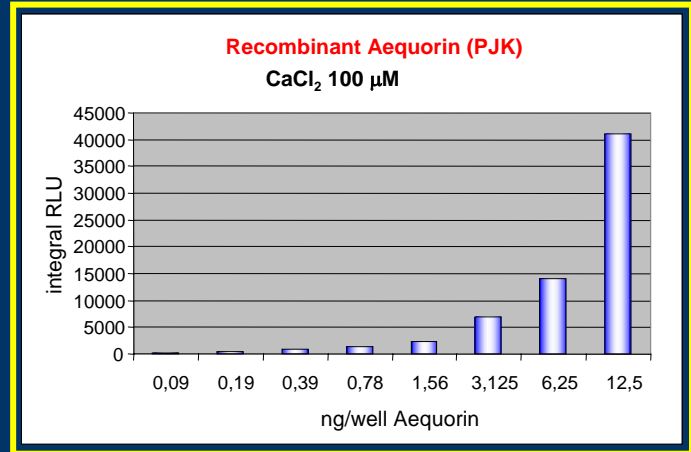
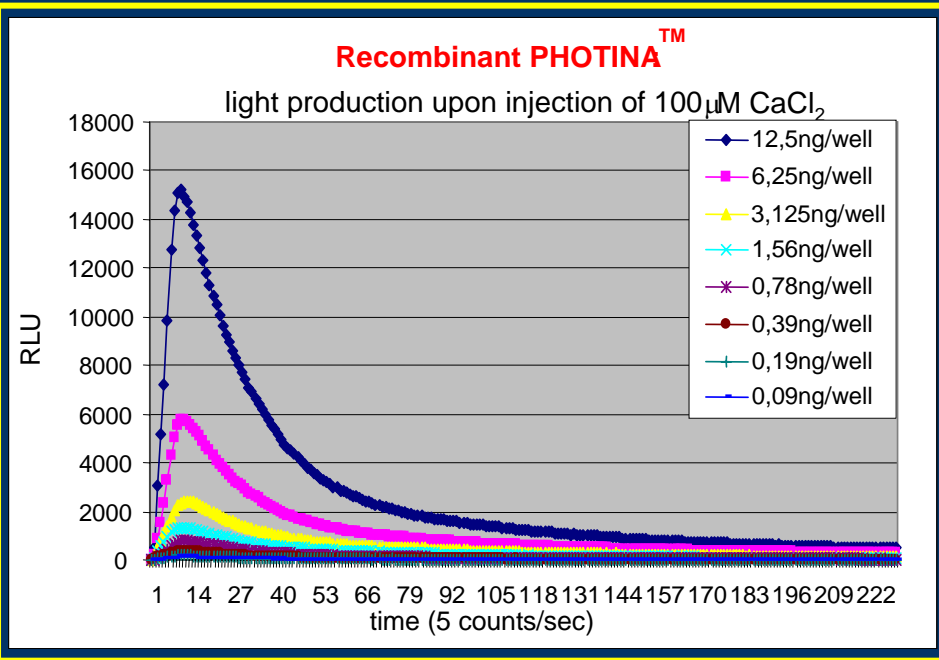
“In vivo”

- CCD Camera
- FLIPR3 Luminescence
- FLIPR TETRA Luminescence



Characteristics and Characterisation of Photina™

Light production : recombinant Photina™



Characteristics and Characterisation of Photina™

- Stably expressed in CHO and HEK cells
- Cytoplasmic expression
- Tagged version to mitochondria and plasma membrane
- Tested for GPCR cell based assays

- CHO mitoPhotina™ CCD camera
 - Histamine3 receptor (384 MTP)
 - Adenosine3 receptor (384 MTP)
 - Fractalkine receptor (1536 MTP)



Characterisation of Photina™: CCD Camera

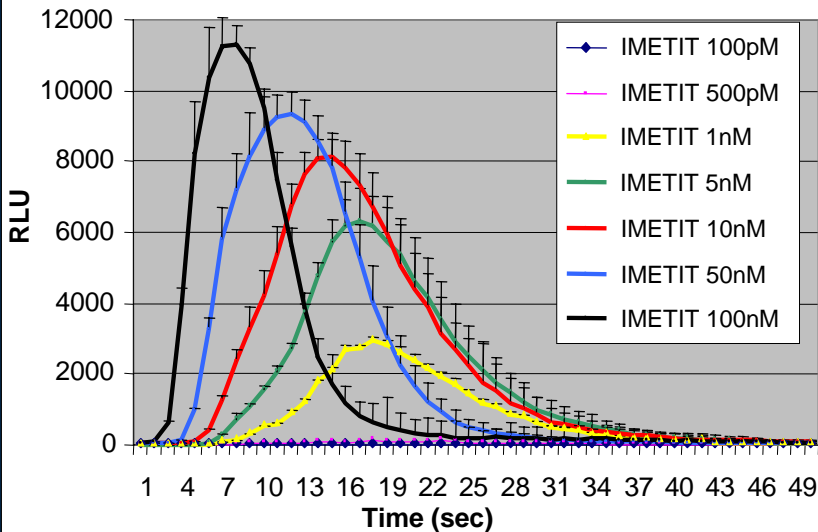
Histamine receptor 3, H3

agonist (IMETIT) dose-response

Imetit

CHO-mito-PHOTINA™/H3

750c/w MTP 384 24h (sensitivity 50%)

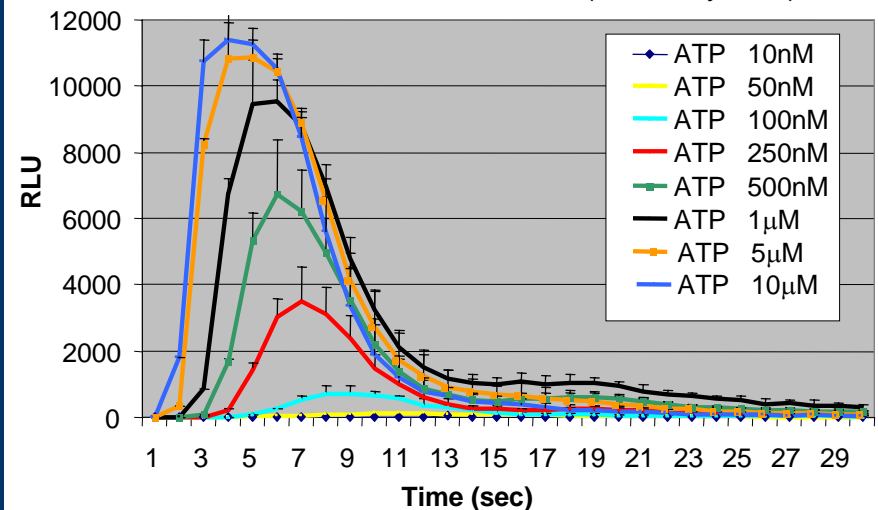


ATP dose-response

ATP

CHO-mito-PHOTINA™/H3

750c/w MTP 384 24h (sensitivity 50%)



- 384 MTP
- 750 cells/well, 24 hrs
- 5 µM coelenterazine
- 3 hrs incubation at 37°C
- 50% CCD Camera sensitivity

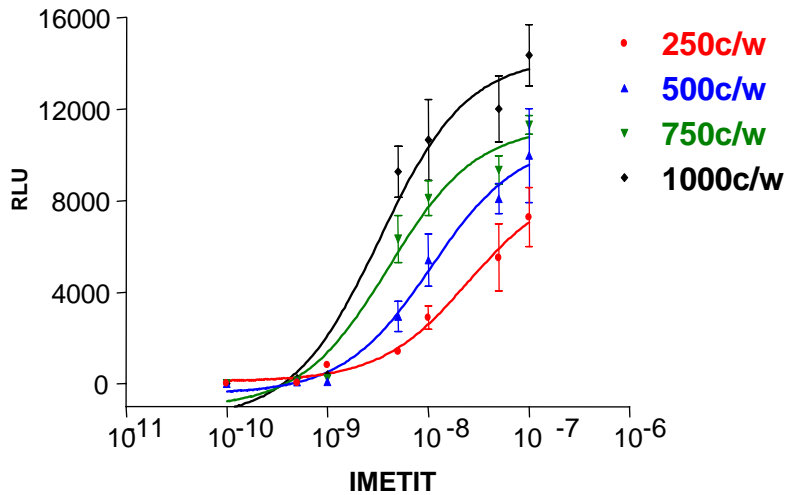


Characteristics and Characterisation of Photina™

Histamine receptor 3, H3

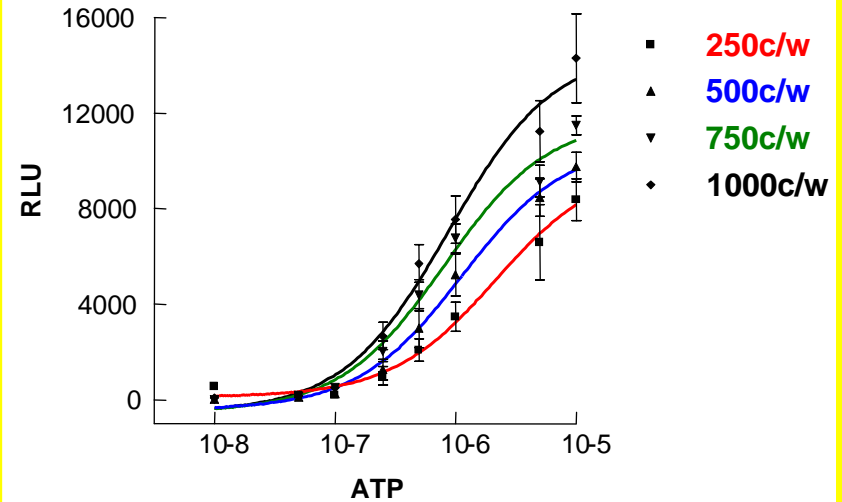
Imetit CHO-mito-PHOTINA™/H3

MTP 384 24h
COELENTERAZINE 5uM



ATP CHO-mito-PHOTINA™/H3

MTP 384 24h
COELENTERAZINE 5uM



EC₅₀

250 c/w	24 nM
500 c/w	11 nM
750 c/w	4 nM
1000 c/w	3,3 nM

EC₅₀

250 c/w	2,6 μM
500 c/w	1,1 μM
750 c/w	0,8 μM
1000 c/w	0,9 μM

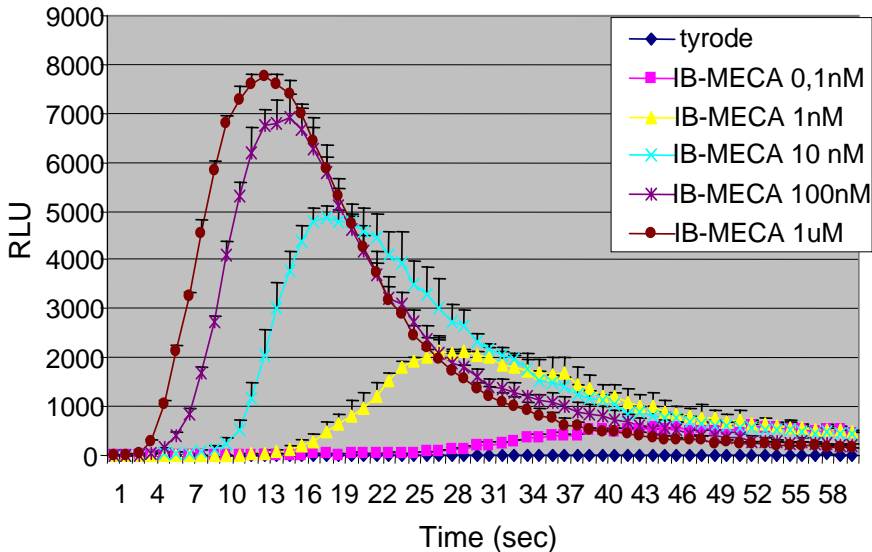


Characteristics and Characterisation of Photina™

Adenosine-3 receptor, A3

agonist (IB-MECA) dose-response

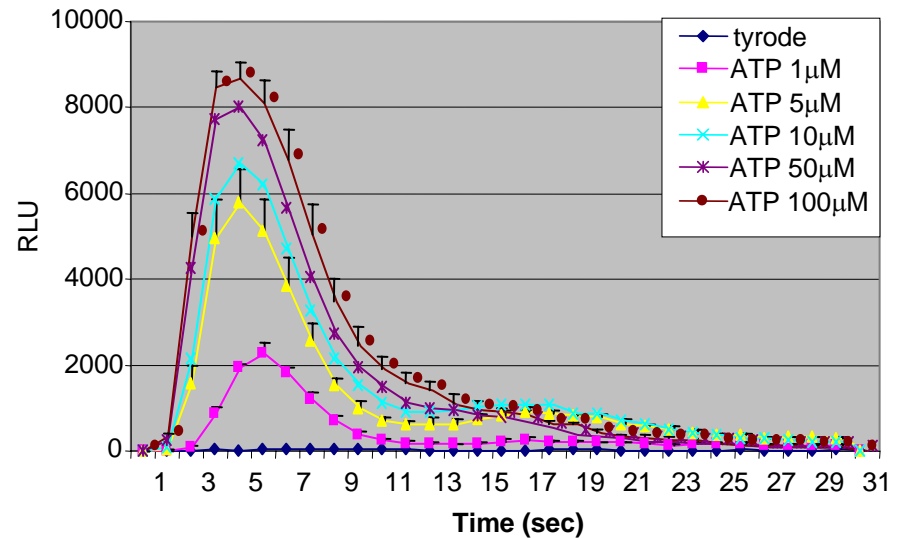
IB-MECA CHO-mito-PHOTINA™/A3



- 384 MTP
- 1000 cells/well, 24 hrs
- 5 μM coelenterazine
- 3 hrs incubation at 37°C
- 50% CCD Camera sensitivity

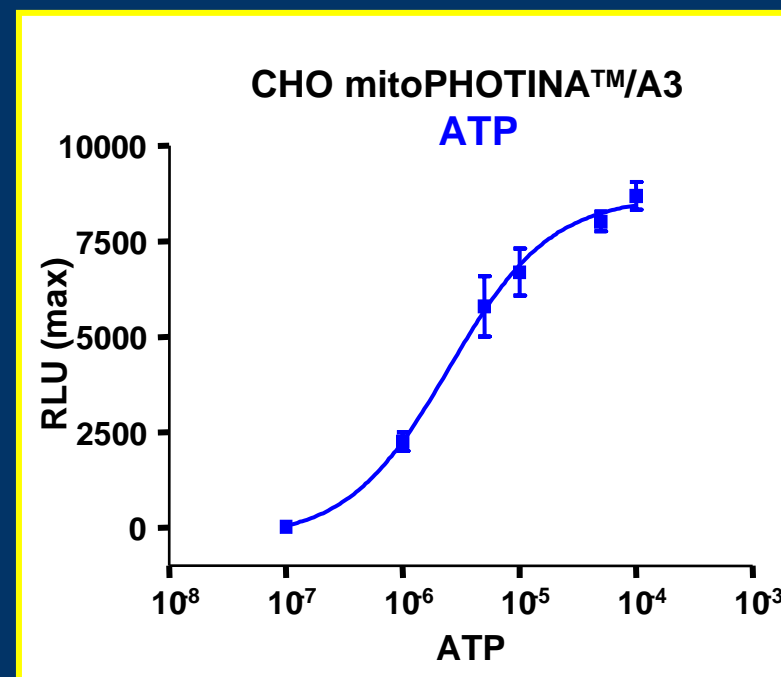
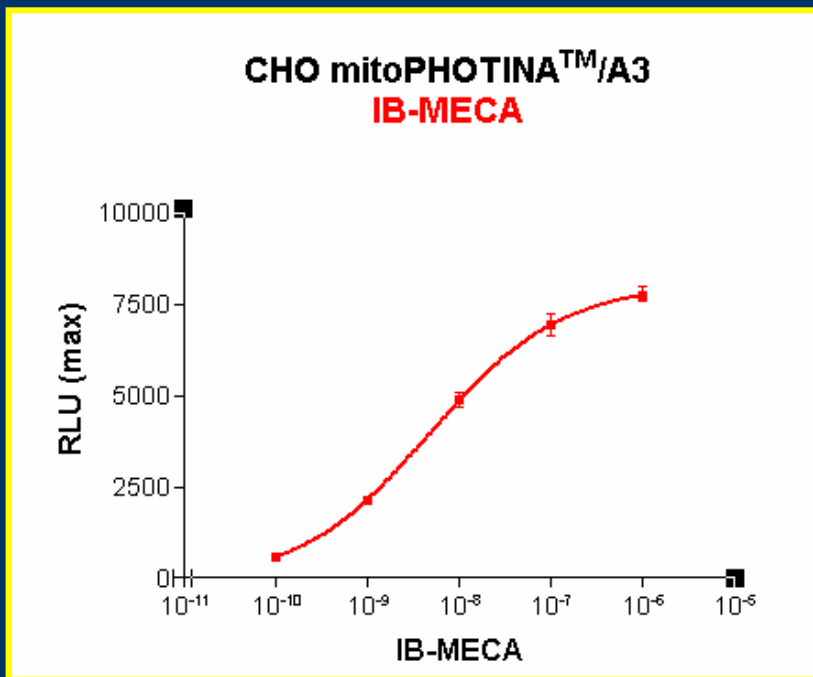
ATP dose-response

ATP CHO-mito-PHOTINA™/A3



Characteristics and Characterisation of Photina™

Adenosine-3 receptor, A3



EC₅₀	R²
4,7 nM	1

- 384 MTP
- 1000 cells/well, 24 hrs
- 5 μM coelenterazine
- 3 hrs incubation at 37°C
- 50% CCD Camera sensitivity

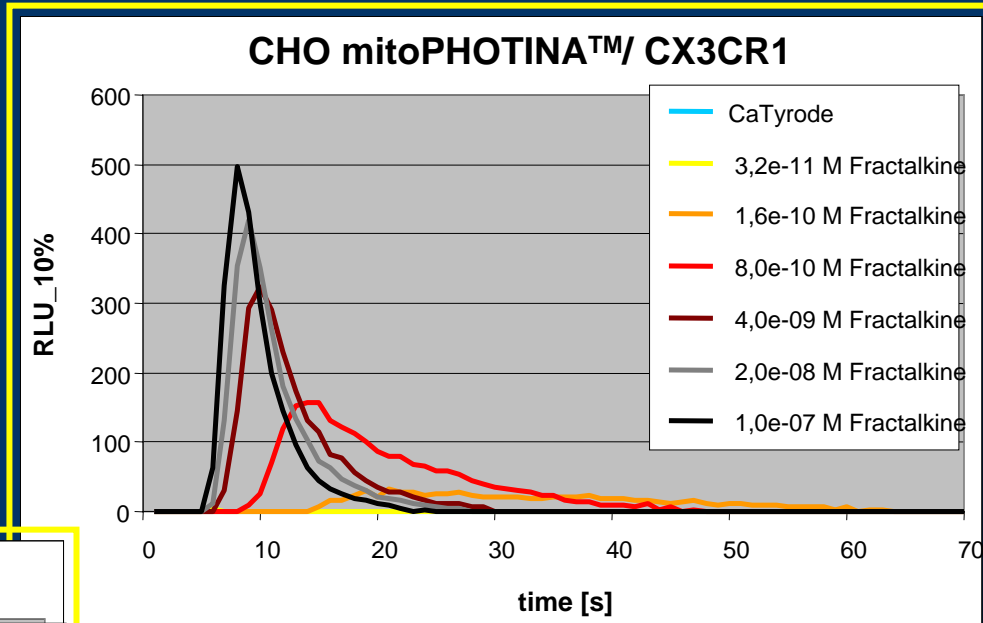
EC₅₀	R²
2,5 μM	0,997



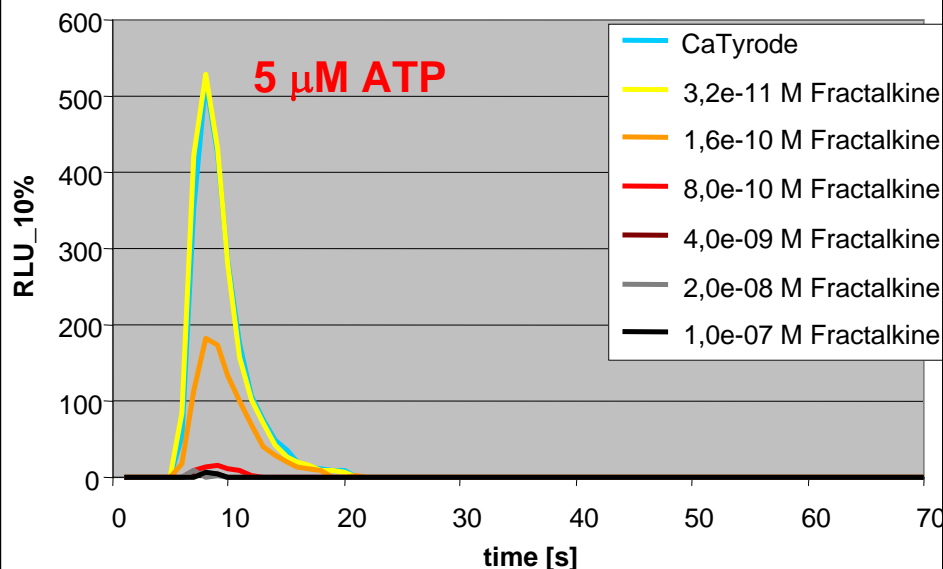
Characteristics and Characterisation of Photina™

1536 well format, CCD camera

- 250 cells/well were plated in 1536 well plate 48 h before the experiment
- Cells were loaded with 2,5 μM coelenterazine for 2 h
- Luminescence was recorded with a CCD camera at 10% sensitivity



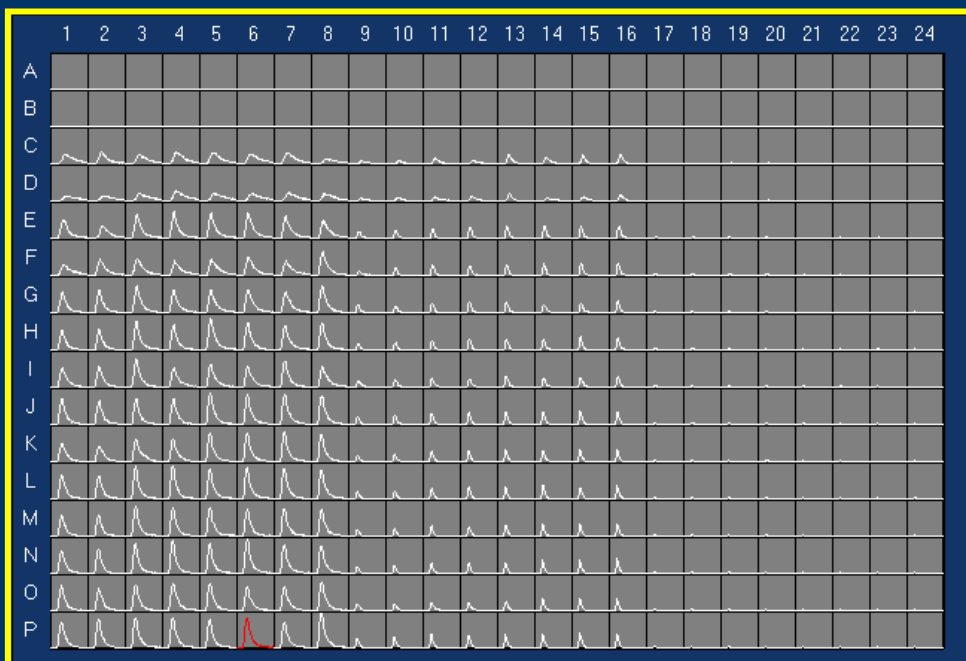
CHO mitoPHOTINA™/ CX3CR1



- Low number of cells/well required
- Low quantity of coelenterazine required
- Good pharmacology of endogenous and exogenous receptors



FLIPR3 & Luminescence



FLIPR3 Settings for Photina™ Assays

The image displays two screenshots of the FLIPR3 software interface, showing the configuration of assay sequences. The left screenshot shows the 'Pre-Assay Steps' tab, and the right screenshot shows the 'Sequences' tab.

Pre-Assay Steps (Left Screenshot):

- Assign Plate - 384 well head
 - Plate Position 1 = Default 384
 - Plate Position 2 = Default 384
 - Plate Position 3 = None
 - Plate Position 4 = None
- Assign Filter for All Imaging
 - Use Filter = Filter 1
- Configure Camera
 - Exposure Length = 0.7
 - Camera Gain = 200
- Manual Bar Code
- Load Plates From Stacker
 - Read Bar Code From = 0
- Close Door
- Check Machine Status
- Pre-Incubate Plates
- Pre-Soak - 384 well head
- Create Document Name
 - Include Date = 1
 - Include User Defined String = 1
 - Include Bar Code = 0
 - Include Experiment Number = 1
 - User Defined String = Axxam_1_addition
- Prompt Note

Sequences (Right Screenshot):

- Aspirate - 384 well head
 - From Plate = 2
 - Height(ul) = 20
 - Volume(ul) = 25
 - Speed(ul/sec) = 20
 - Hold Volume(ul) = 1
- Put Tips in Target Well - 384 head
 - Height(ul) = 30
- Baseline Imaging
 - Image Interval (sec) = 1
 - # of Images = 5
- Dispense - 384 well head
 - Tips Already in Well? = 1
 - Target Plate = 1
 - Height(ul) = 30
 - Speed(ul/sec) = 25
 - Expel Volume(ul) = 1
 - Mix Volume(ul) = 0
 - Number of Mix Cycles = 0
 - Pause for Next Move(sec) = 120
- Wash Tips
- Wash Tips - Original Washer
- First Interval
 - Image Interval (sec) = 1
 - # of Images = 60
- Second Interval
 - Image Interval(sec) = 3
 - # of Images = 20
- Automated Tip Unload
- Close Reagent Head

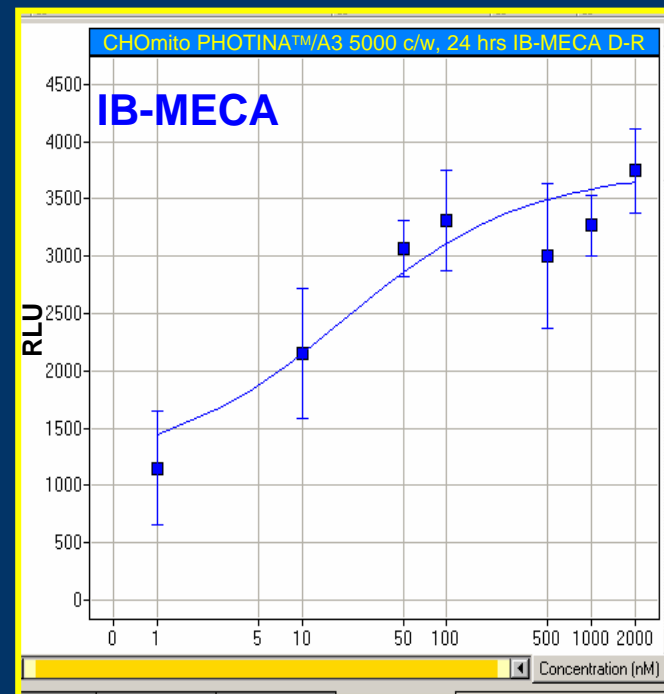
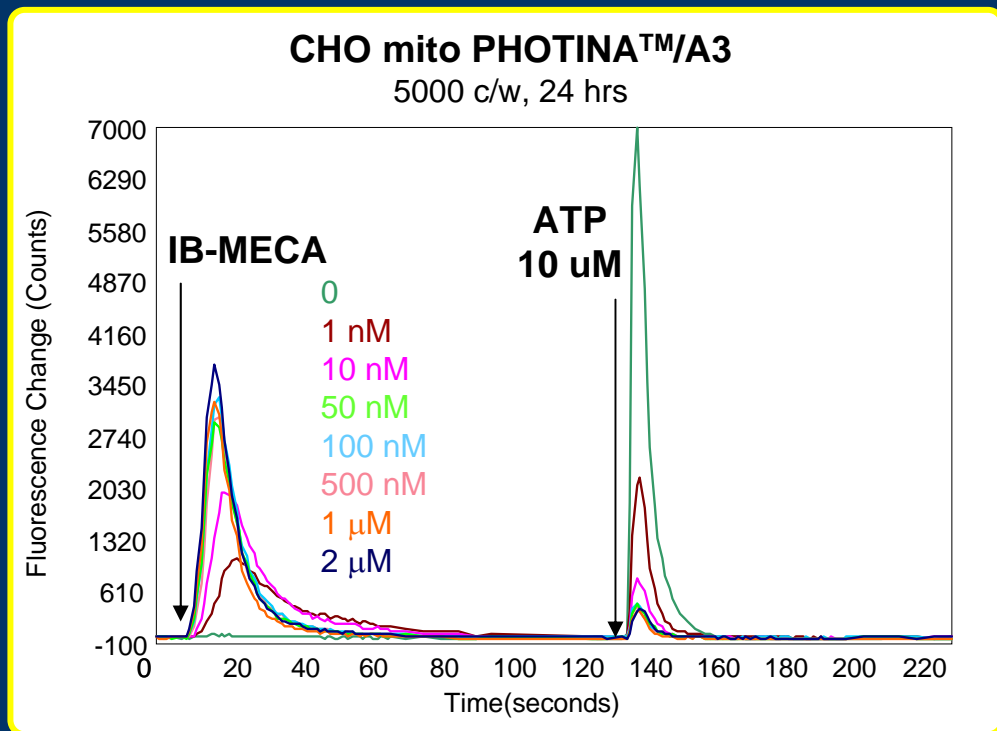
Standard protocol for Photina™ detection

- 384 white wall clear bottom plates
- cell plating 24 hrs before the experiment
- medium removal
- addition of tyrode + coelenterazine 25µl/well
- incubation 4 hrs at 37°C
- experiment run at FLIPR3: compounds (2X) injection in tyrode buffer (25µl/well)



Adenosine Receptor 3, A3

Agonist (IB-MECA) dose-response



- 384 MTP
- 24 hrs
- 5 μ M coelenterazine
- 4 hrs incubation at 37°C

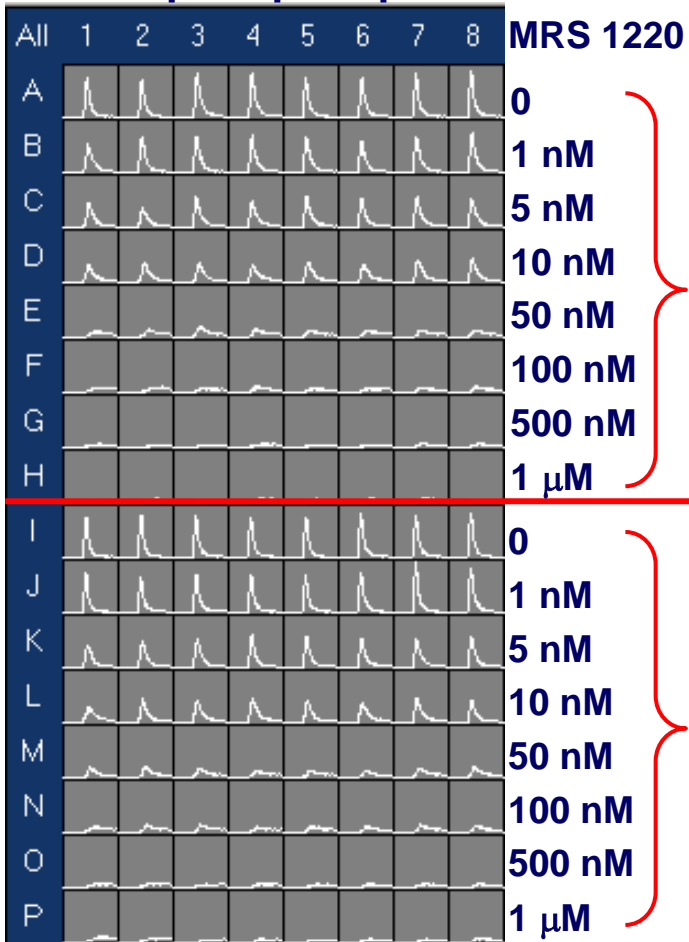
	EC₅₀	R²
5000 c/w	19 nM	0,886



Adenosine Receptor 3, A3

Antagonist (MRS 1220) dose-inhibition

c/w: 2500 | 5000 | 7500 | 10000

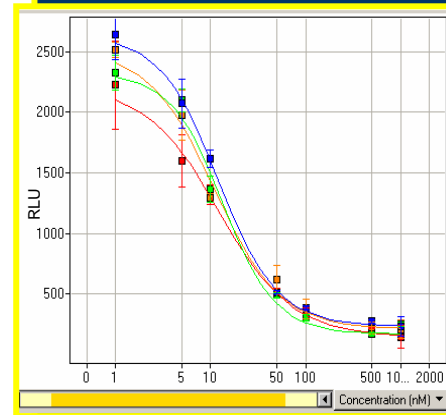


50 nM IB-MECA
in the presence of
increasing
concentration of
antagonist

1 μM IB-MECA
in the presence of
increasing
concentration of
antagonist

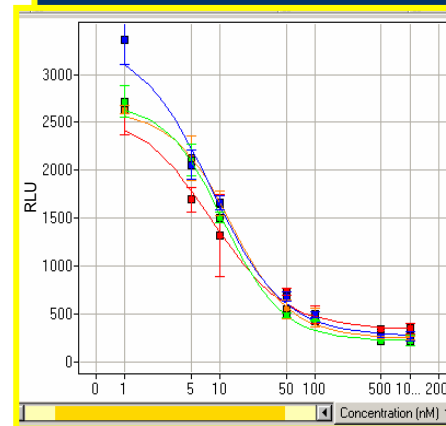
Range = (-200, 3700)

Mini Graphs



	IC₅₀	R²
2,500 c/w	12 nM	0,985
5,000 c/w	11 nM	0,99
7,500 c/w	13 nM	0,989
10,000 c/w	12 nM	0,999

2500 c/w
5000 c/w
7500 c/w
10000 c/w

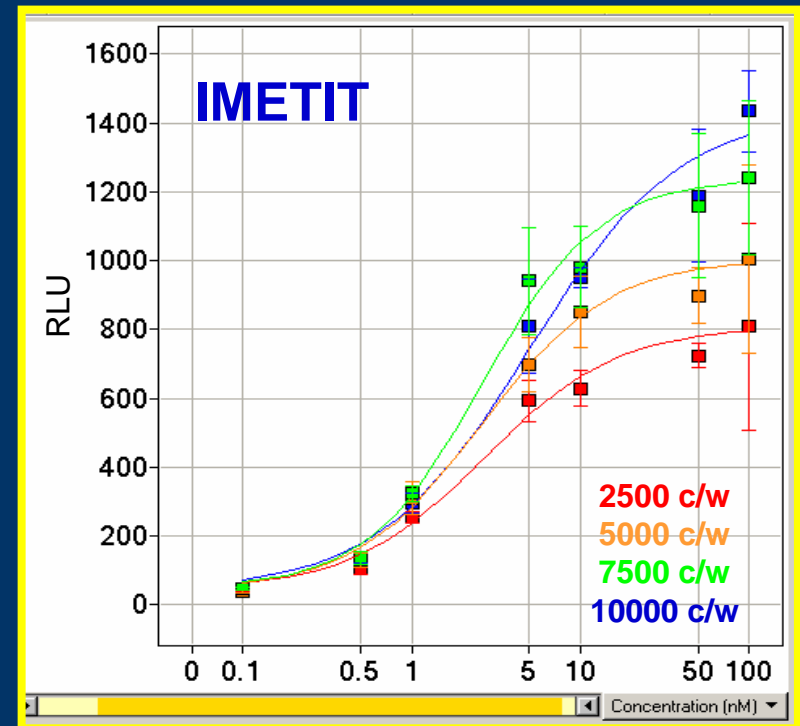
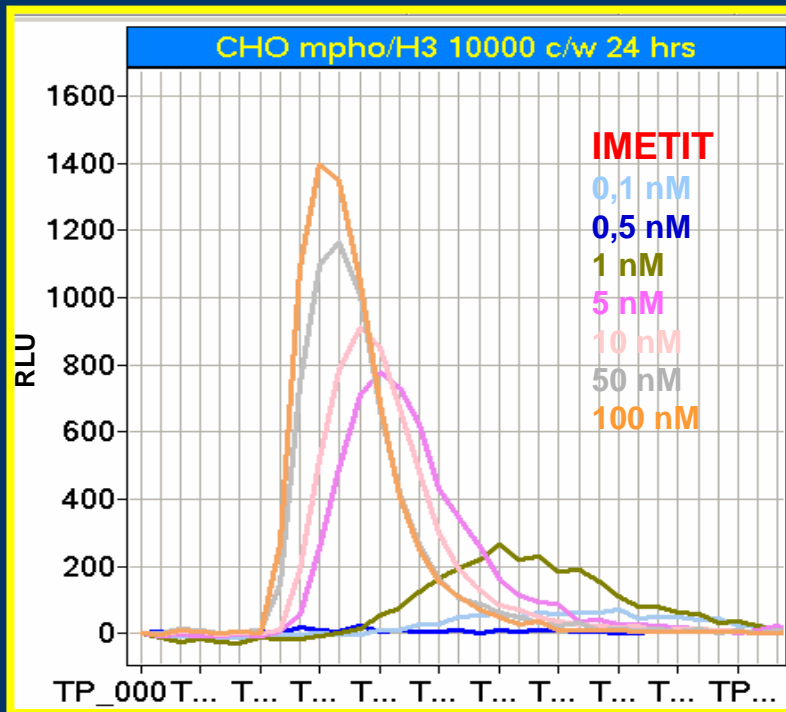


	IC₅₀	R²
,2500 c/w	8 nM	0,985
5,000 c/w	13 nM	0,998
7,500 c/w	12 nM	0,997
10,000 c/w	8 nM	0,987



Histamine Receptor 3, H3

Agonist (IMETIT) dose-response



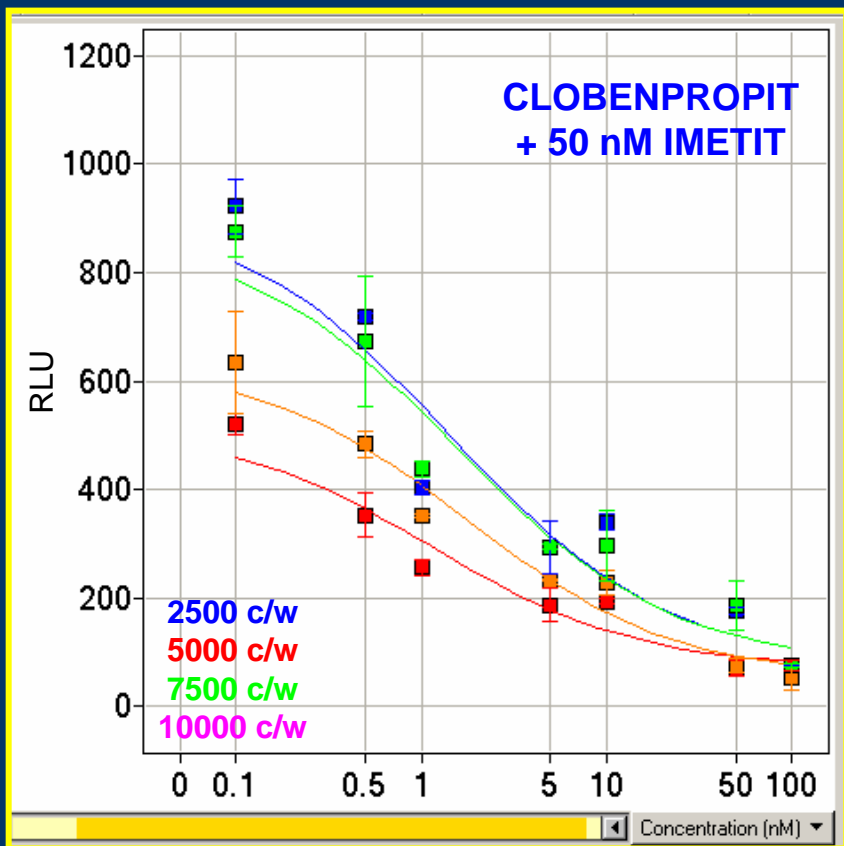
- 384 MTP
- 24 hrs
- 5 μ M coelenterazine
- 4 hrs incubation at 37°C

	EC_{50}	R^2
2,500 c/w	2,7 nM	0,985
5,000 c/w	2,5 nM	0,991
7,500 c/w	2,6 nM	0,99
10,000 c/w	4,9 nM	0,984



Histamine Receptor 3, H3

Antagonist (CLOBENPROPIT) dose-inhibition



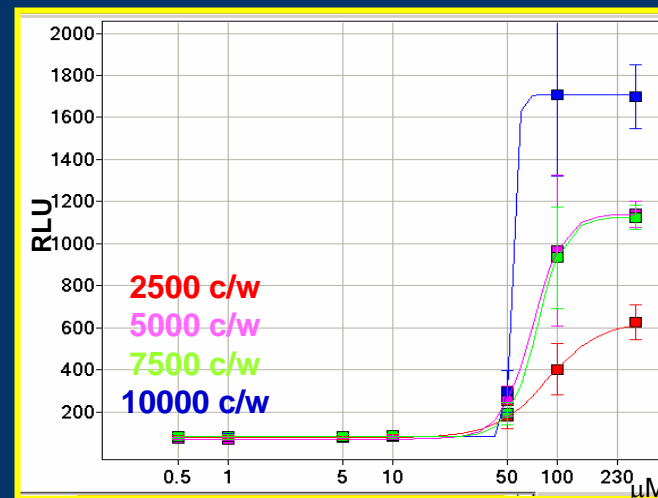
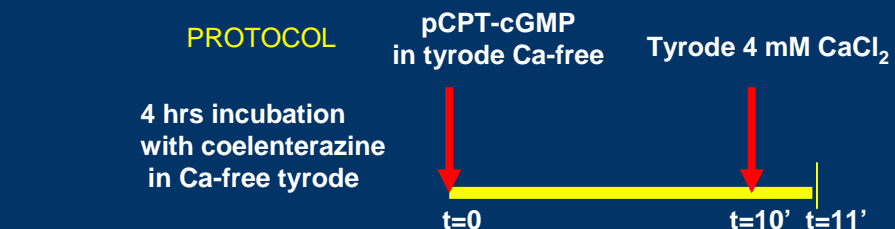
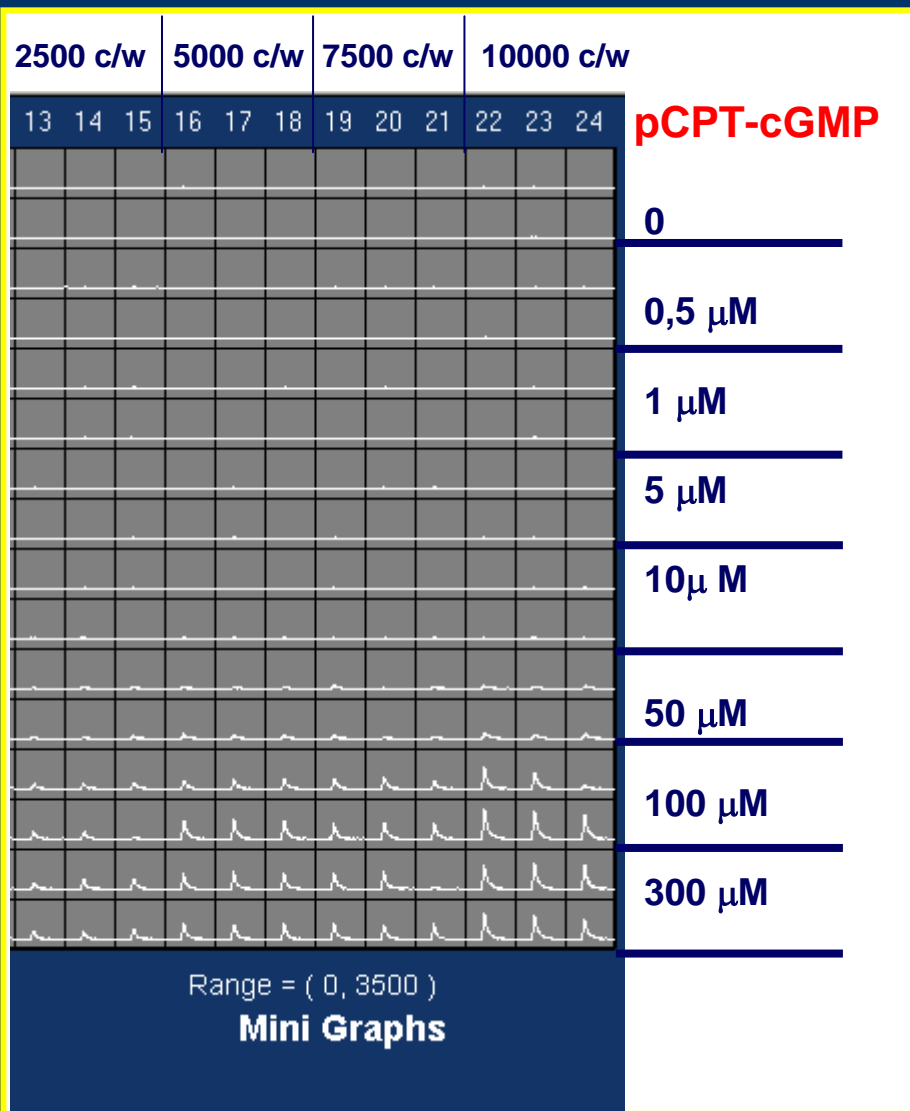
- 384 MTP
- 24 hrs
- 5 μ M coelenterazine
- 4 hrs incubation at 37°C

	IC_{50}	R^2
2,500 c/w	1,1 nM	0,937
5,000 c/w	1,7 nM	0,961
7,500 c/w	1,5 nM	0,939
10,000 c/w	1,4 nM	0,905



Cyclic Nucleotide-gated (CNG) Channel

Agonist (pCPT-cGMP) dose-response

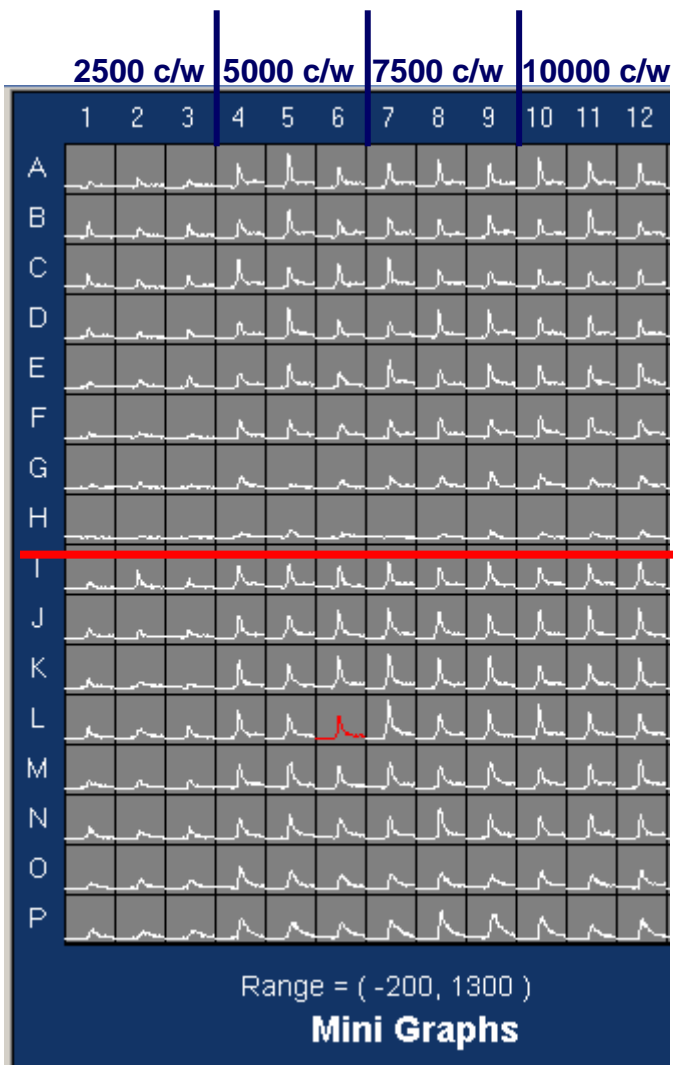


	EC₅₀	R²
2,500 c/w	86 μM	0,999
5,000 c/w	70 μM	1
7,500 c/w	75 μM	1
10,000 c/w	53 μM	1



Vanilloid Receptor 1 (VR1)

Antagonist (CAPSAZEPINE) dose-inhibition



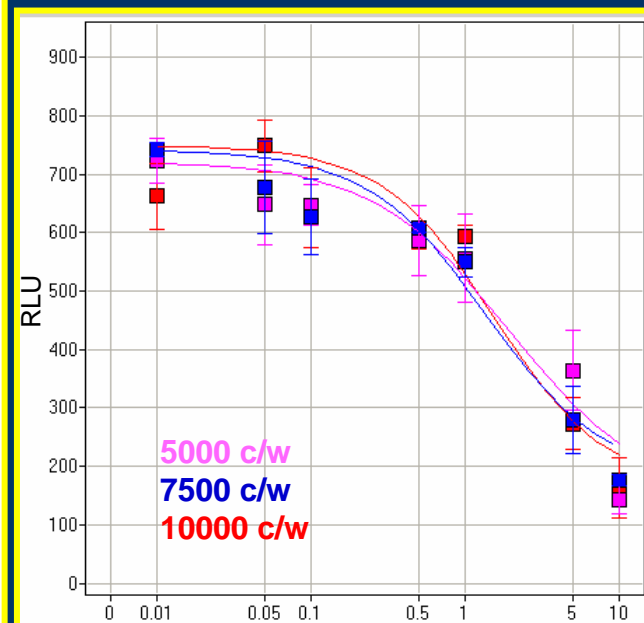
CZ

0
0,01 μM
0,05 μM
0,1 μM
0,5 μM
1 μM
5 μM
10 μM

5 μM CAPSAICIN
in the presence of
increasing
concentration of
capsazepine (CZ)

0
0,01 μM
0,05 μM
0,1 μM
0,5 μM
1 μM
5 μM
10 μM

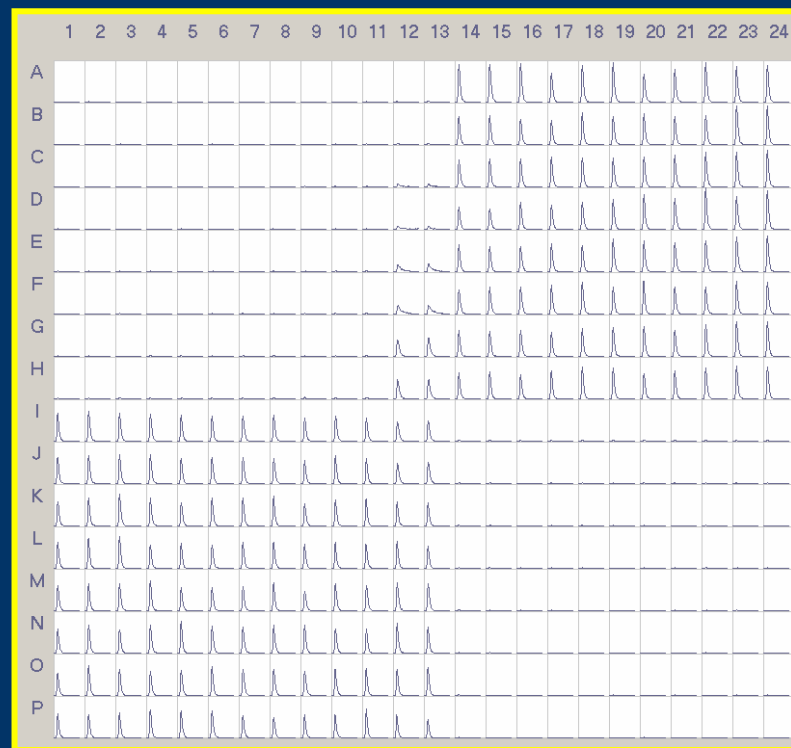
50 μM CAPSAICIN
in the presence of
increasing
concentration of
capsazepine (CZ)



	IC₅₀	R²
5,000 c/w	1,9 μM	0,921
7,500 c/w	1,5 μM	0,918
10,000 c/w	1,3 μM	0,945



FLIPR TETRA & Luminescence



FLIPR TETRA Settings for Photina™ Assays

Setup Read Mode:

Read Mode Name	Exc. Wlength	Em. Wlength	Gain	Exp. Time	Exc. Intensity
<input checked="" type="checkbox"/> Read_Mode_1	NONE	NONE	200	0.50	0
<input type="checkbox"/> Read_Mode_2	470_495	515_575	80	0.40	50
<input type="checkbox"/> Read_Mode_3	470_495	515_575	80	0.40	50
<input type="checkbox"/> Read_Mode_4	470_495	515_575	80	0.40	50

Edit mode

Assign Plate to Position:

Position	Plate Name	BarCode Expect	Bar Code List
Read Plate	default384	NO	
Source Plate 1	default384	NO	
Source Plate 2	NONE	NO	
Source Plate 3	NONE	NO	

Transfer Fluid:

Fluid transfer type: Read

Aspirate	Plate	Volume	Height	Speed	TipSpeed	HoldVol	Quadrant	Done
<input checked="" type="checkbox"/> Aspirate	Source Plate 1	25.0	30.0	20.0	4.0	1.0	NONE	NO
<input type="checkbox"/> Aspirate	Source Plate 1	25.0	4.0	20.0	10.0	1.0	NONE	NO
<input type="checkbox"/> Aspirate	Source Plate 1	25.0	4.0	20.0	10.0	1.0	NONE	NO
<input type="checkbox"/> Aspirate	Source Plate 1	25.0	4.0	20.0	10.0	1.0	NONE	NO

Put tips in well before read Edit Aspirate

Dispense	Plate	Volume	Height	Speed	ExpeVol	Pause	TipSpeed	Quadrant	Done
<input checked="" type="checkbox"/> Dispense	Read Plate	25.0	30.0	25.0	1.0	0	4.0	Quadrant 1	NO
<input type="checkbox"/> Dispense	Read Plate				1.0	0	10.0	Quadrant 1	NO
<input type="checkbox"/> Dispense	Read Plate	25.0	40.0	20.0	1.0	0	10.0	Quadrant 1	NO
<input type="checkbox"/> Dispense	Read Plate	25.0	40.0	20.0	1.0	0	10.0	Quadrant 1	NO

First Interval

Read interval: sec

Number of reads:

Number of reads before dispense:

Second Interval

Read interval: sec

Number of reads:

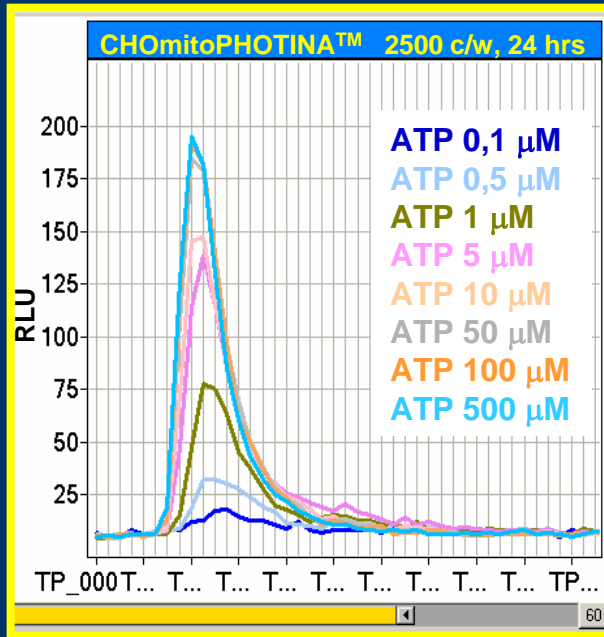
Standard protocol for Photina™ detection

- 384 white wall clear bottom plates
- cell plating 24 hrs before the experiment
- medium removal
- addition of tyrode + coelenterazine 25µl/well
- incubation 4 hrs at 37°C
- experiment run at FLIPR TETRA: compounds (2X) injection in tyrode buffer (25µl/well)

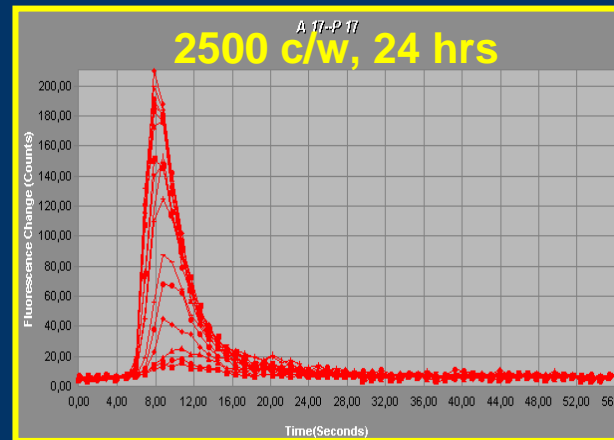
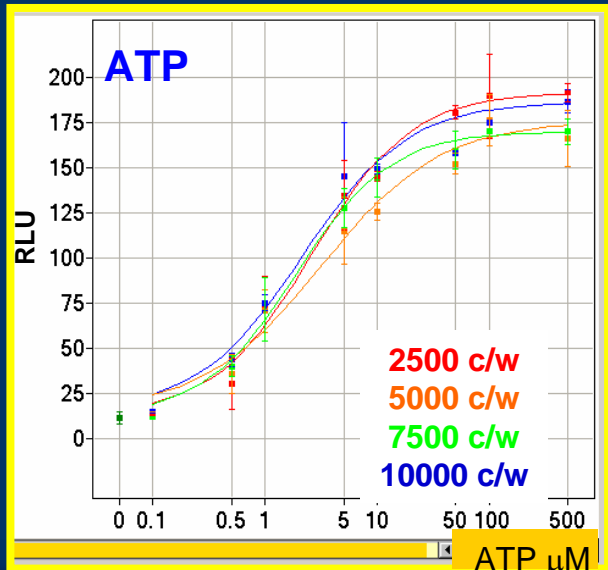


Endogenous P2Y Receptors

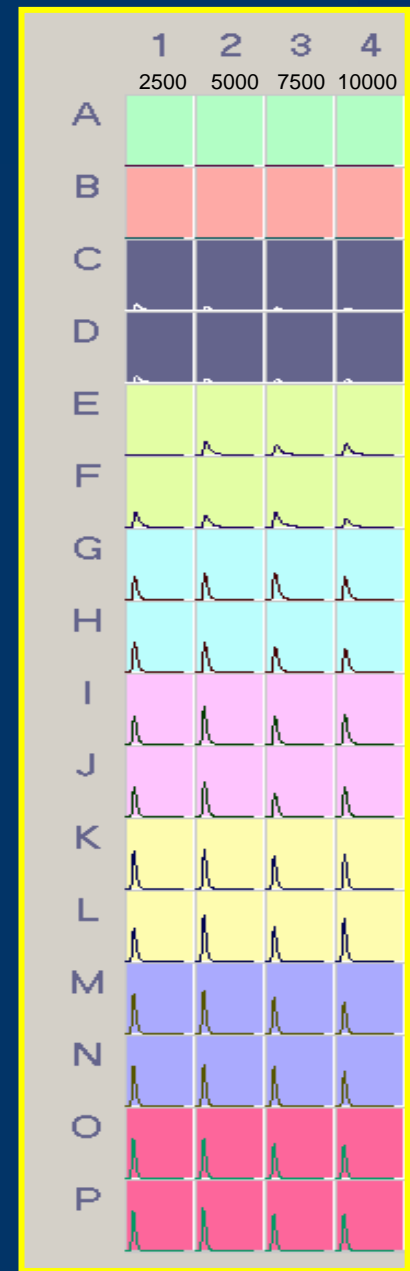
ATP response



- 384 MTP
- 24 hrs
- 5 μM coelenterazine
- 4 hrs incubation at 37°C

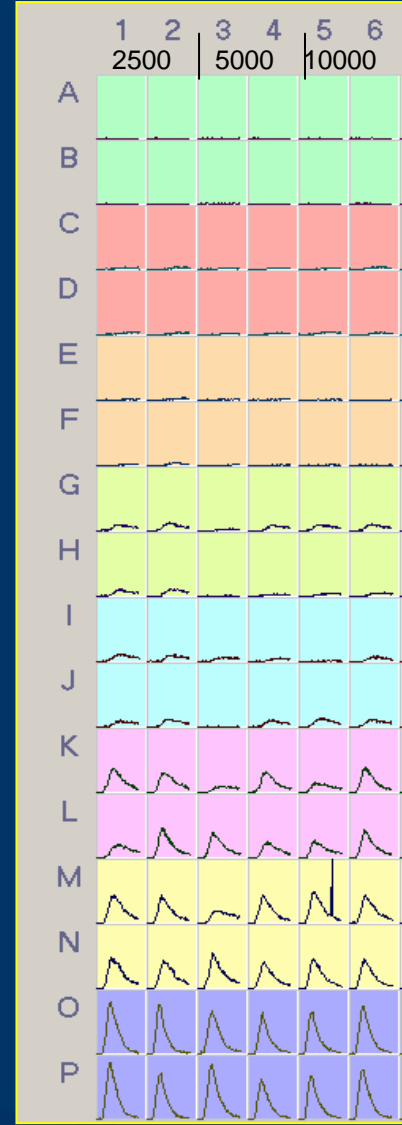
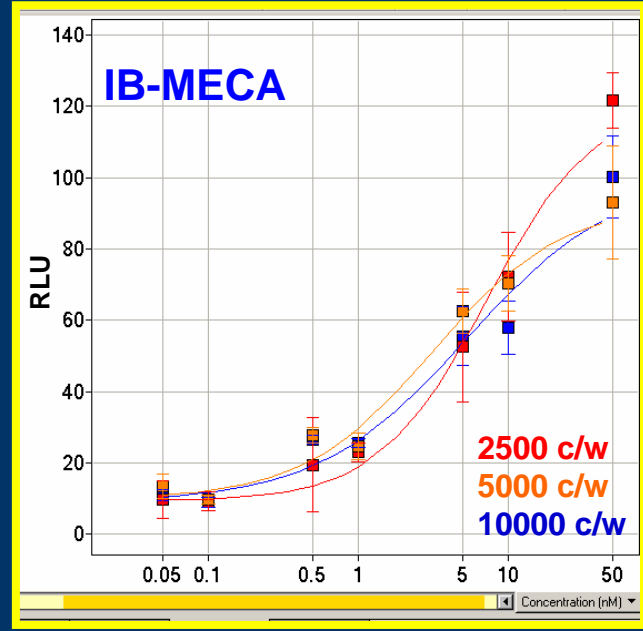
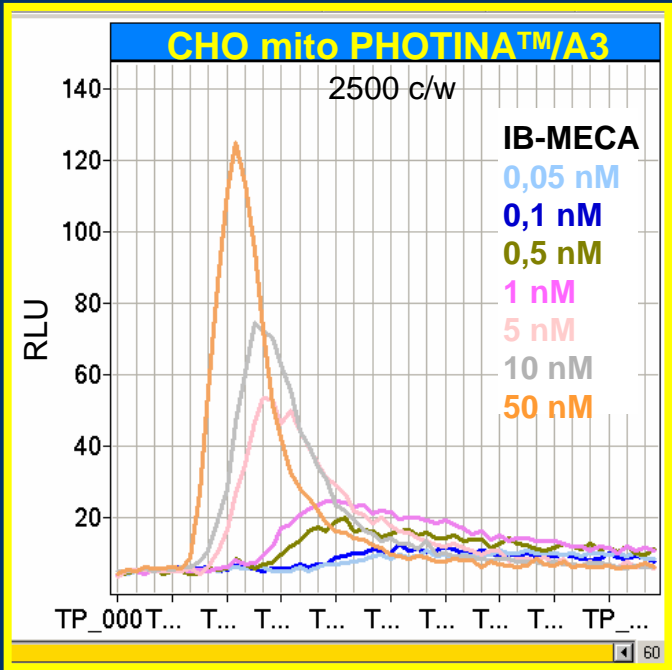


	EC ₅₀	R ²
2,500 c/w	2,6 μM	0,988
5,000 c/w	3,1 μM	0,979
7,500 c/w	1,8 μM	0,995
10,000 c/w	2,1 μM	0,974



Adenosine Receptor 3, A3

Agonist (IB-MECA) dose-response



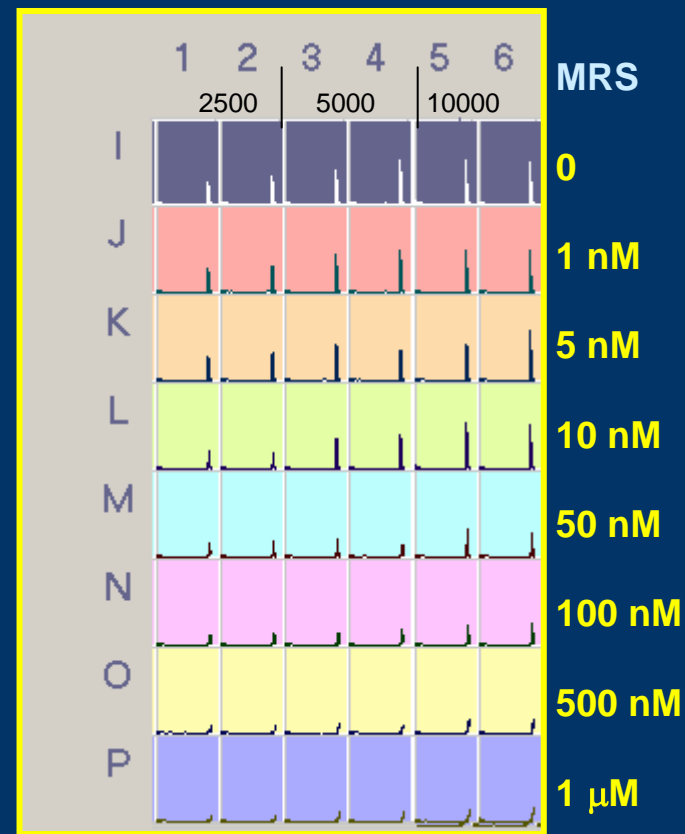
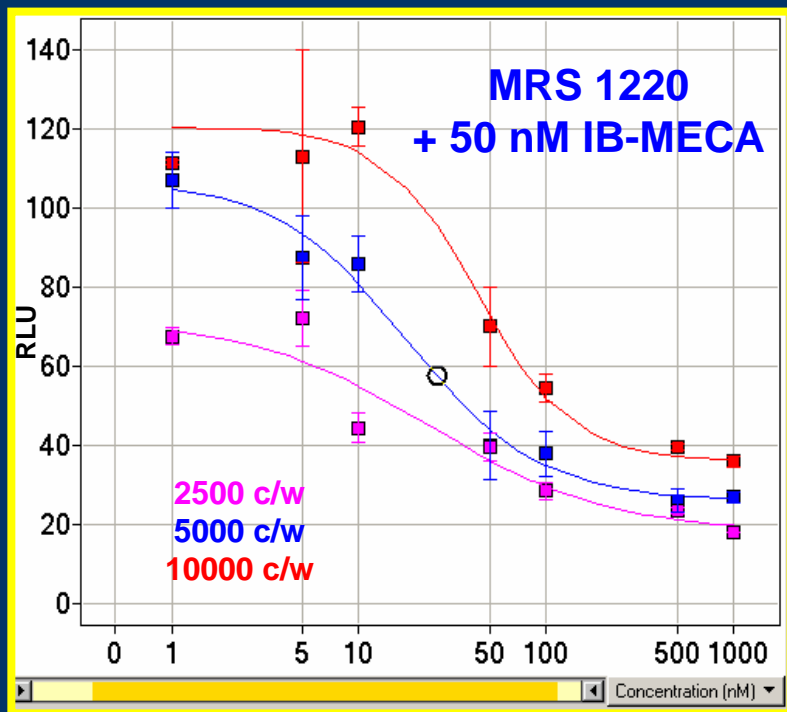
- 384 MTP
- 24 hrs
- 5 μ M coelenterazine
- 4 hrs incubation at 37°C

	EC₅₀	R²
2,500 c/w	7,1 nM	0,984
5,000 c/w	3,1 nM	0,981
10,000 c/w	5,2 nM	0,958



Adenosine Receptor 3, A3

Antagonist (MRS 1220) dose-inhibition



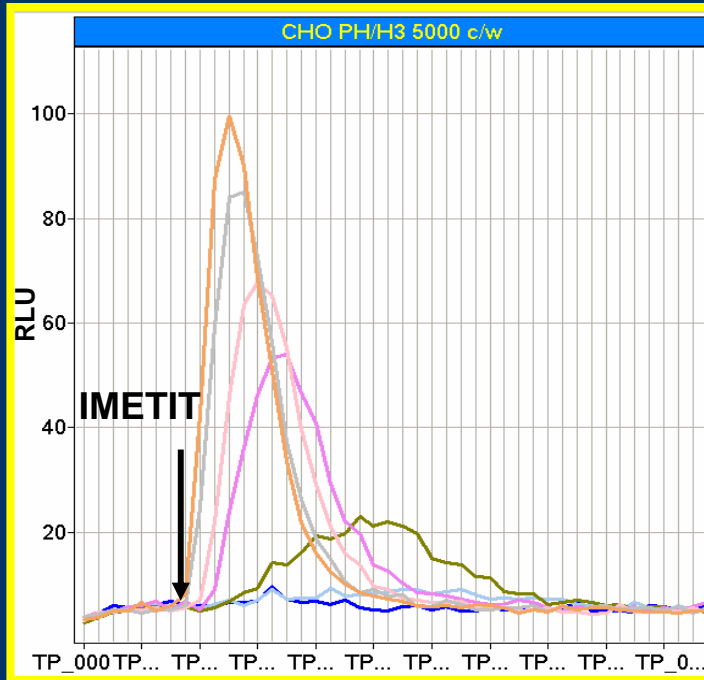
	IC₅₀	R²
2,500 c/w	23 nM	0,907
5,000 c/w	18 nM	0,986
10,000 c/w	43 nM	0,979

- 384 MTP
- 24 hrs
- 5 μM coelenterazine
- 4 hrs incubation at 37°C



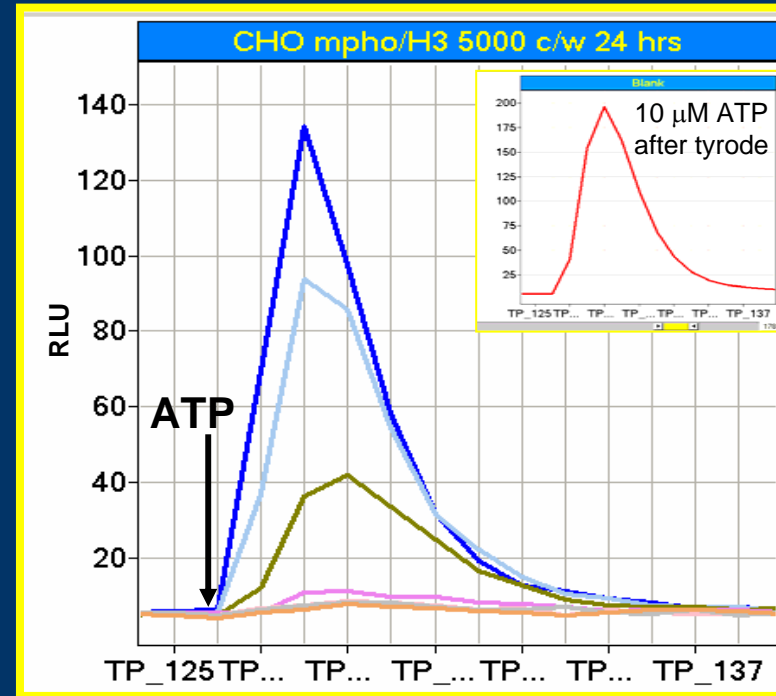
Histamine Receptor 3, H3

Agonist (IMETIT) and residual ATP responses



IMETIT
0,1 nM
0,5 nM
1 nM
5 nM
10 nM
50 nM
100 nM

Residual ATP response:
10 μ M ATP has been injected
10' after a D-R to IMETIT



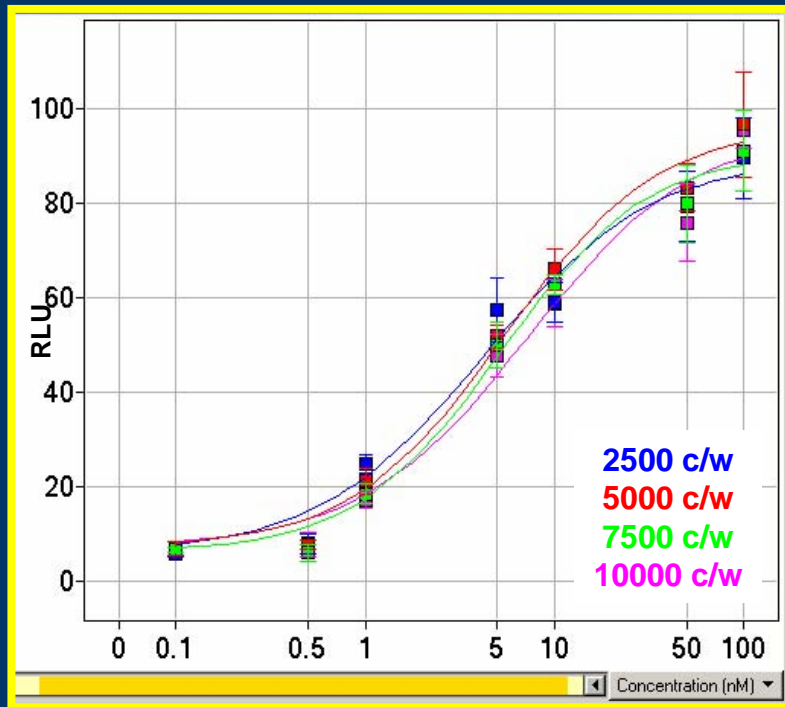
- 384 MTP
- 24 hrs
- 5 μ M coelenterazine
- 4 hrs incubation at 37°C



Histamine Receptor 3, H3

Agonist (IMETIT) pharmacology

agonist (IMETIT) dose-response

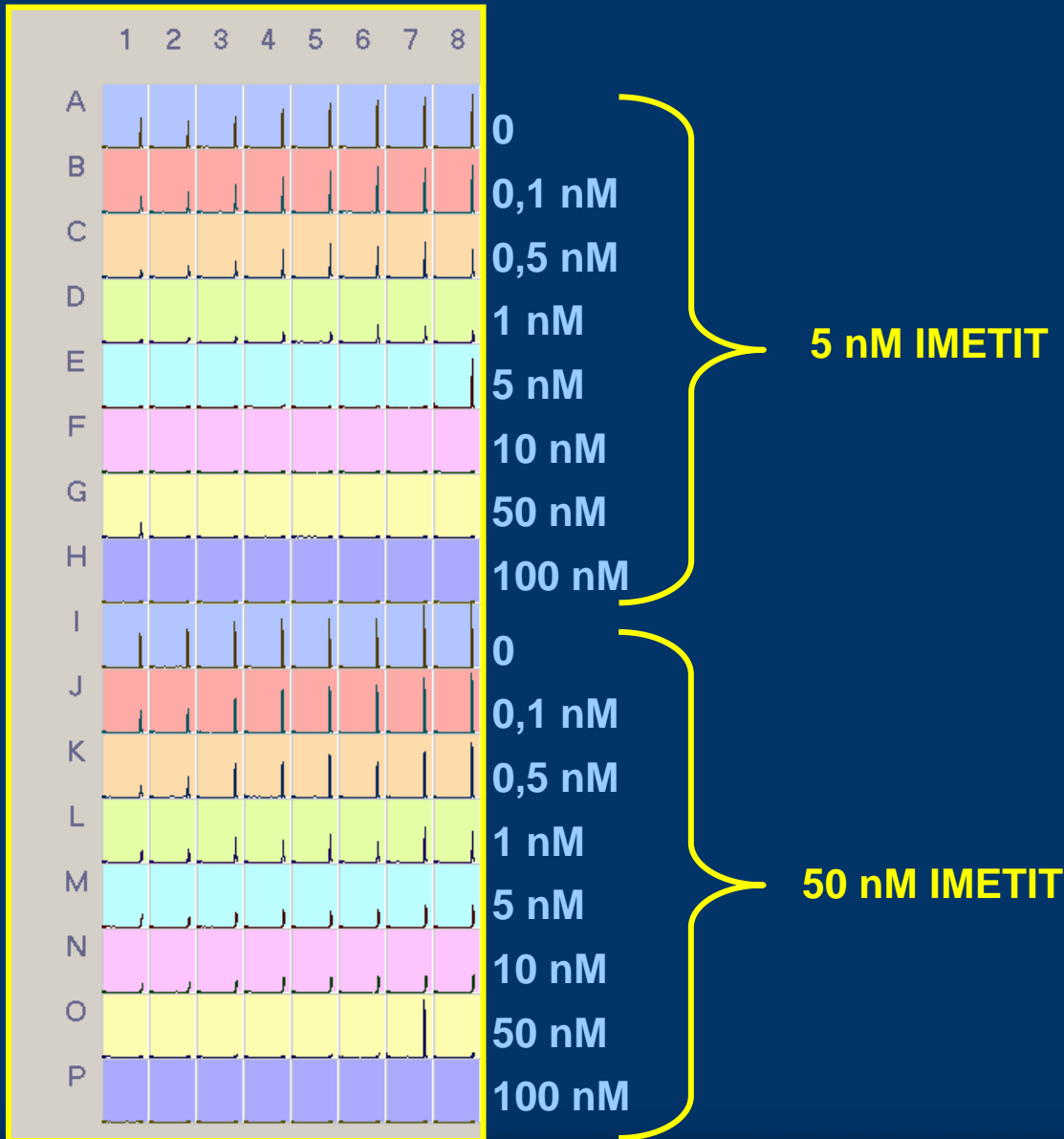


	EC_{50}	R^2
2,500 c/w	4,2 nM	0,978
5,000 c/w	5,4 nM	0,989
7,500 c/w	5,2 nM	0,99
10,000 c/w	7,1 nM	0,978

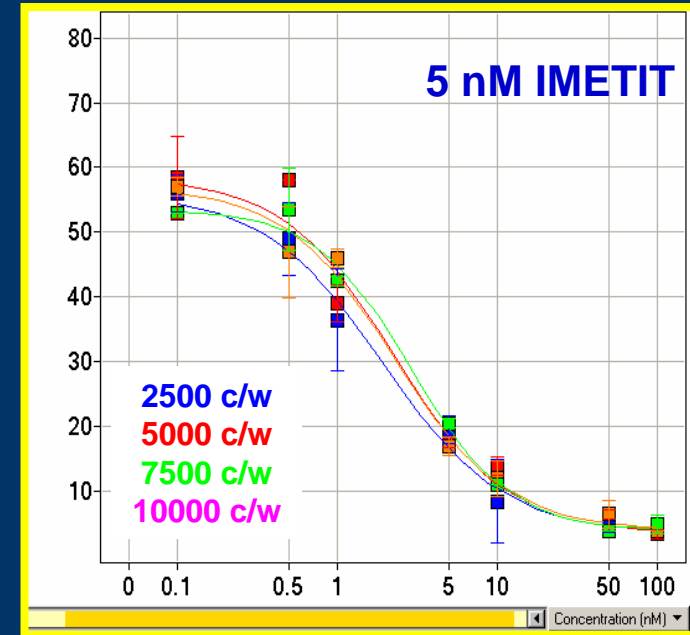


Histamine Receptor 3, H3

Antagonist (CLOBENPROPIT) pharmacology



antagonist (CLOBENPROPIT) dose-inhibition



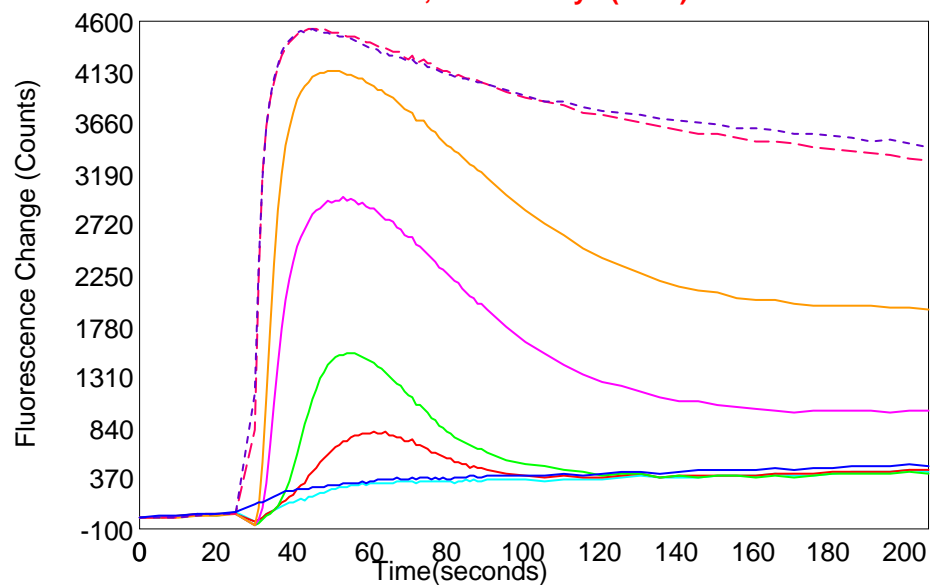
	IC_{50}	R^2
2,500 c/w	2,3 nM	0,974
5,000 c/w	2,3 nM	0,992
7,500 c/w	2,9 nM	0,993
10,000 c/w	1,9 nM	0,991



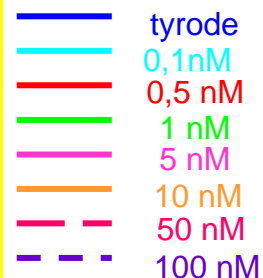
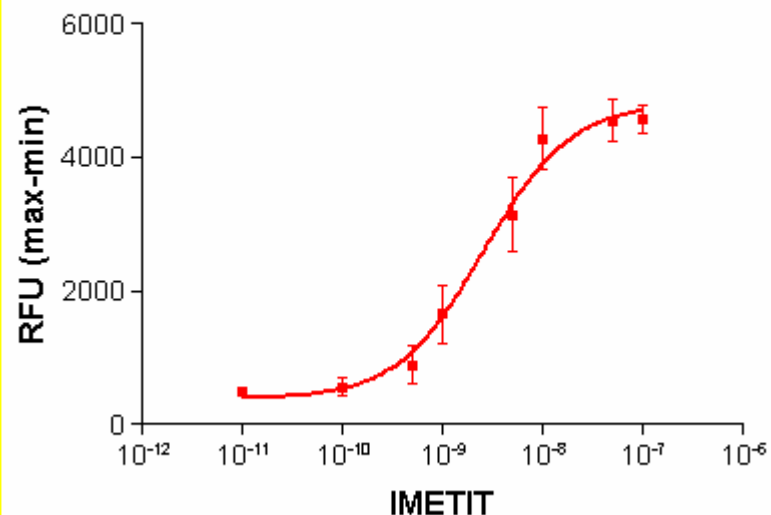
FLIPR_FLUORESCENCE

Histamine receptor 3, H3
IMETIT D-R

CHO mPHOTINA™/H3,
IMETIT D-R, Calcium dye (MDC)



CHO mPhotina™
IMETIT D-R

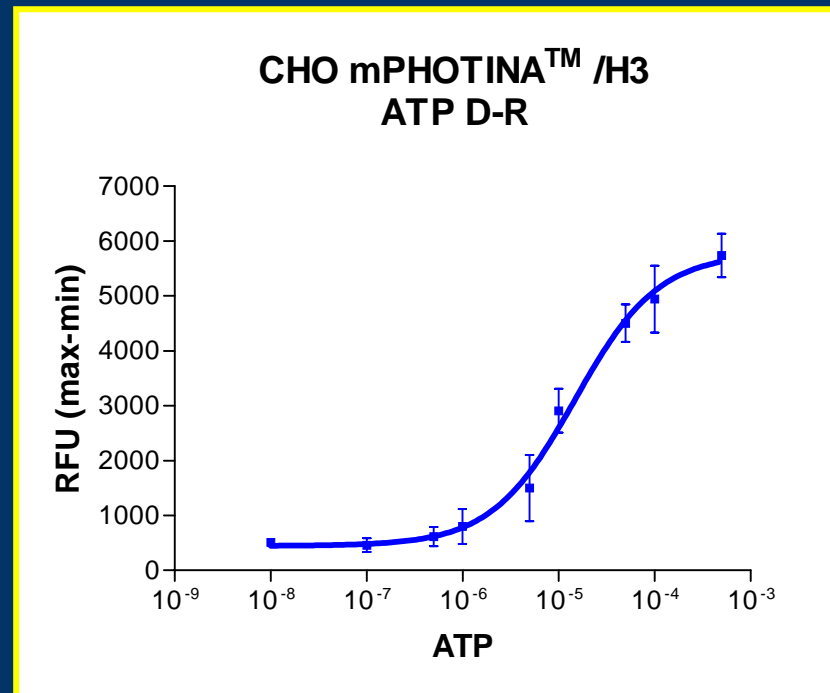
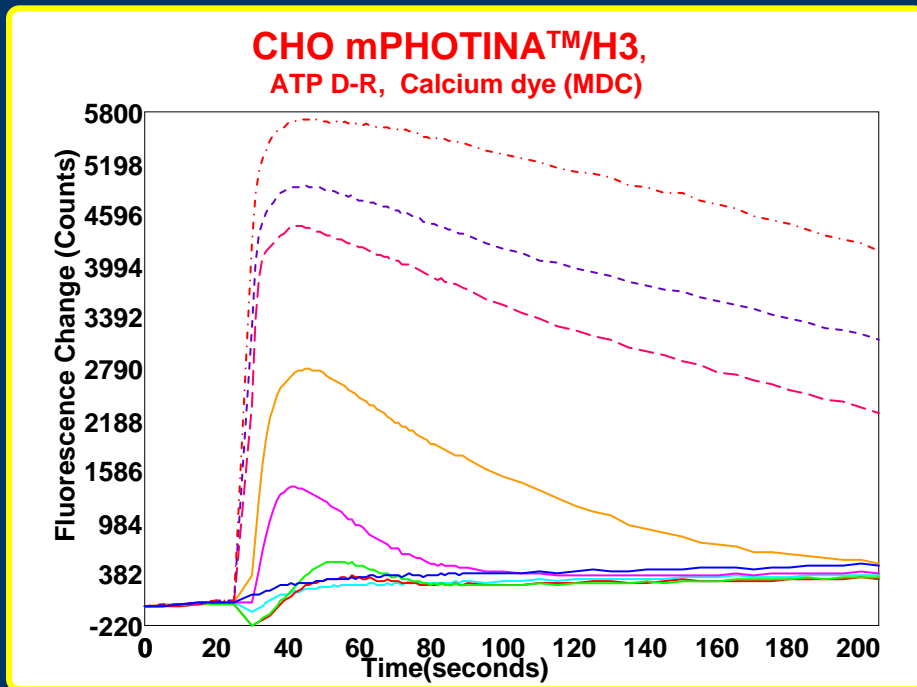


	EC ₅₀	R ²
10,000 c/w	2,6 nM	0,989



FLIPR_FLUORESCENCE

Histamine receptor 3, H3
ATP D-R



- tyrode
- 0,1 μ M
- 0,5 μ M
- 1 μ M
- 5 μ M
- 10 μ M
- 50 μ M
- 100 μ M
- 500 μ M

EC_{50}	R^2
10,000 c/w 14 μ M	0,993



EC₅₀ Comparison

HISTAMINE RECEPTOR 3

EC ₅₀	FLIPR3	FLIPR _{TETRA}	CCD Camera	Fluorescence
2,500 c/w	2,7 nM	4,2 nM	250 c/w 24 nM	
5,000 c/w	2,5 nM	5,4 nM	500 c/w 11 nM	
7,500 c/w	2,6 nM	5,2 nM	750 c/w 4 nM	
10,000 c/w	4,9 nM	7,1 nM	1,000 c/w 3,3 nM	10000 c/w 2,6 nM

ADENOSINE RECEPTOR 3

EC ₅₀	FLIPR3	FLIPR _{TETRA}	CCD Camera
2,500 c/w		7,1 nM	1000 c/w 4,7 nM
5,000 c/w	19 nM	3,1 nM	
10,000 c/w		5,2 nM	

ENDOGENOUS ATP RESPONSE

EC ₅₀	FLIPR _{TETRA}	CCD Camera
2,500 c/w	2,6 μM	250 c/w 2,6 μM
5,000 c/w	3,1 μM	500 c/w 1,1 μM
7,500 c/w	1,8 μM	750 c/w 0,8 μM
10,000 c/w	2,1 μM	1,000 c/w 0,9 μM

Fluorescence
10,000 c/w 14 μM



Photina™ : Concluding Remarks

- interesting alternative for the measurement of intracellular calcium increase,
- stably expressed in mammalian cells, no cytotoxicity shown,
- targeting to specific intracellular compartment,
- suitable for use in HTS and uHTS (CCD Camera, FLIPR3, FLIPR TETRA),
- low number of cells/well needed for HTS,
- higher luminescent signal compared with other known photoproteins,
- low background, excellent signal-to-noise ratio.



Acknowledgements

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