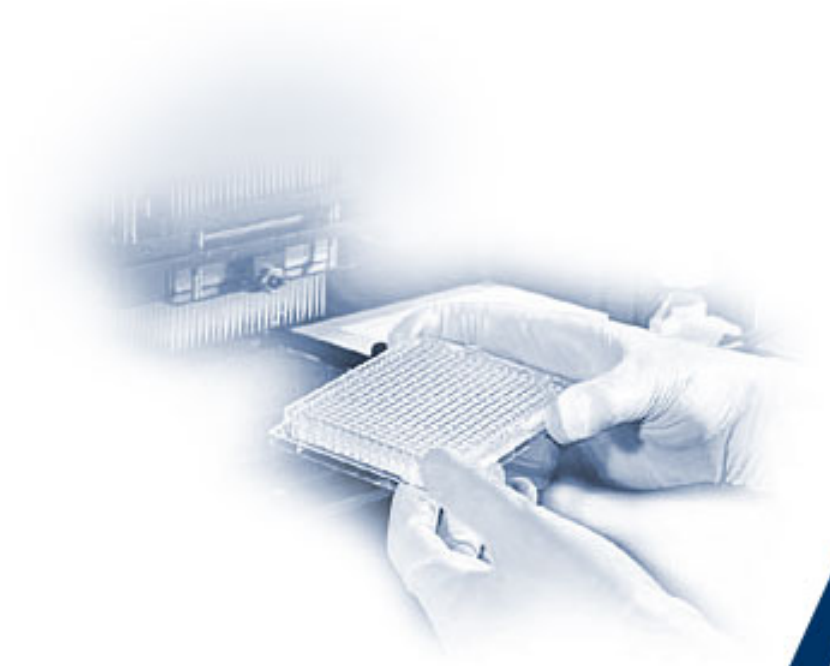


Photina™: an improved Ca2+-sensitive photoprotein

Michela Stucchi, Ph.D

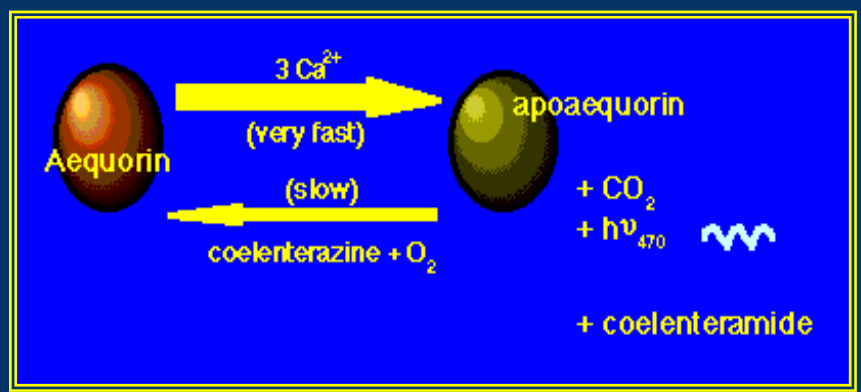
8th International Drug Discovery Conference
June 1-4, 2004
Berkeley, California

Topics of the Presentation

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

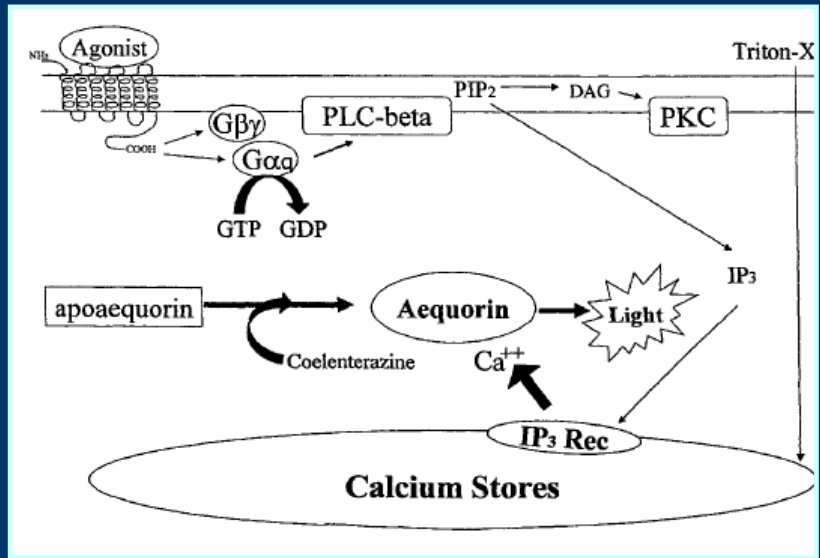
Bioluminescence

- 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

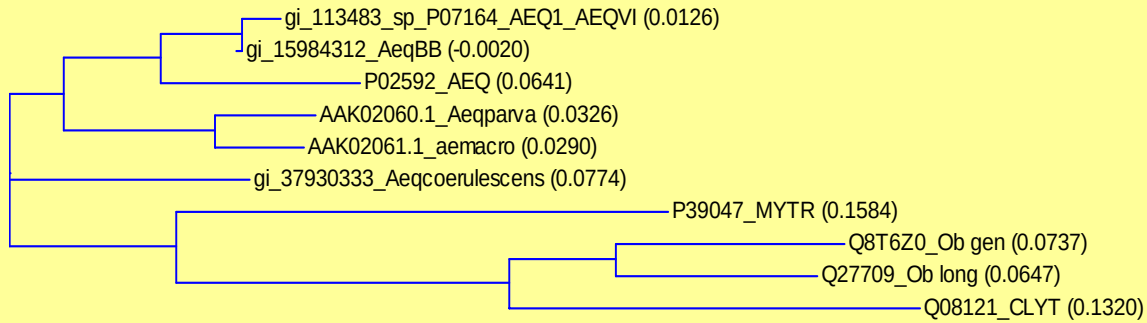
Cloning of new photoproteins

- 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 cystein residues,
- has an overall sequence similarity at the amino acid level of 70% to Aequorin.

Characteristics and Characterisation of Photina™

"In vitro"

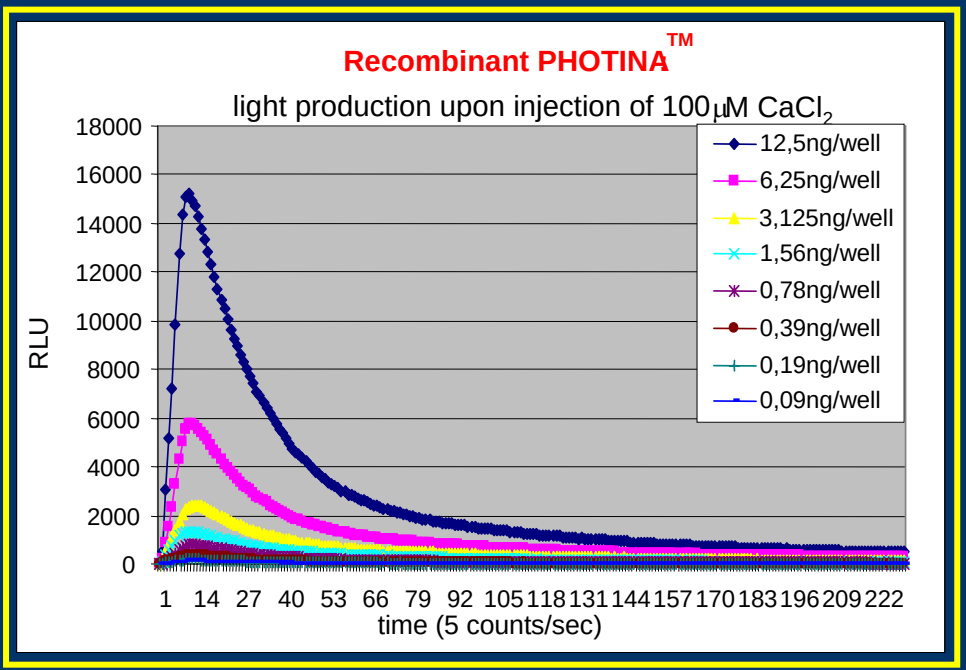
- Light production

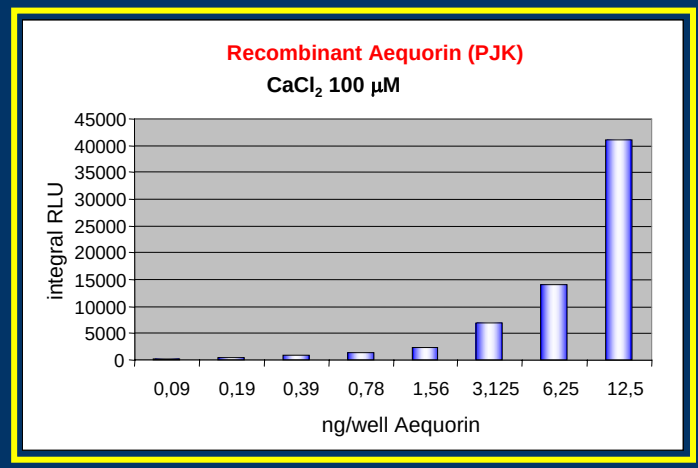
"In vivo"

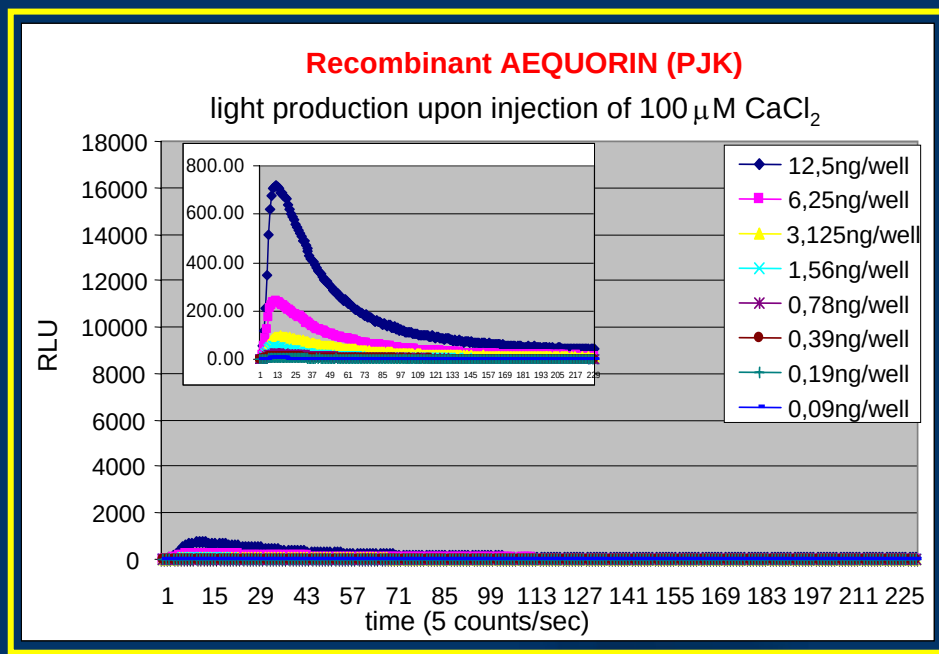
- CCD Camera
- FLIPR3 Luminescence
- FLIPR TETRA Luminescence

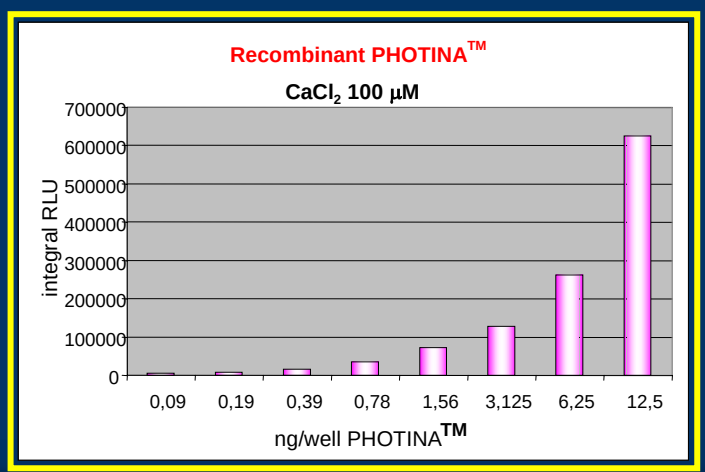
Characteristics and Characterisation of Photina™

Light production : recombinant Photina™









Characteristics and Characterisation of Photina™

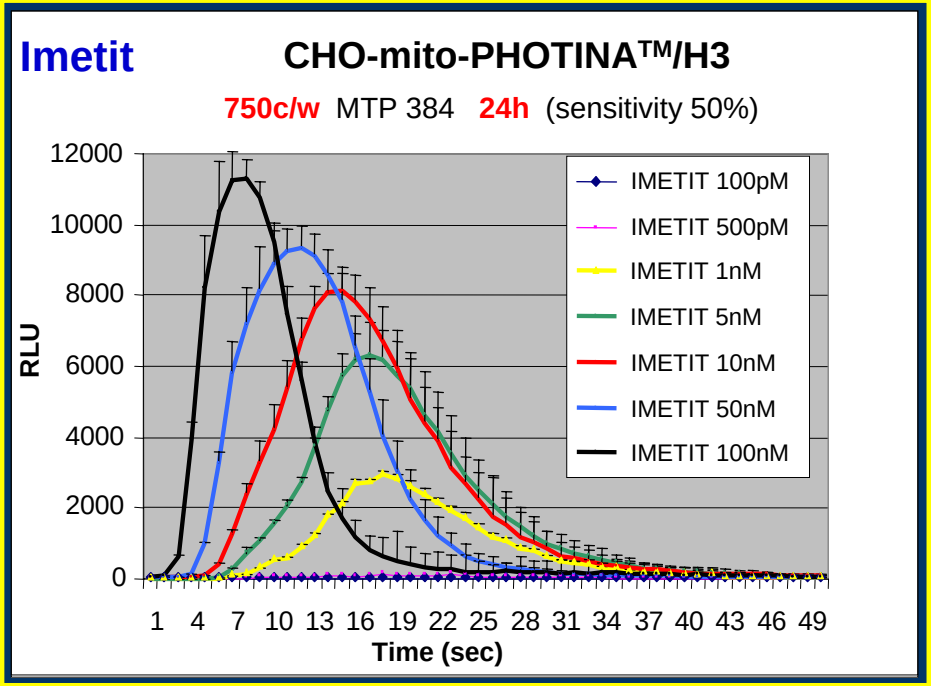
- Stably expressed in CHO and HEK cells
- Cytoplasmic expression
- Tagged version to mitochondria and plasma membrane
- Tested for GPCR cell based assays

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

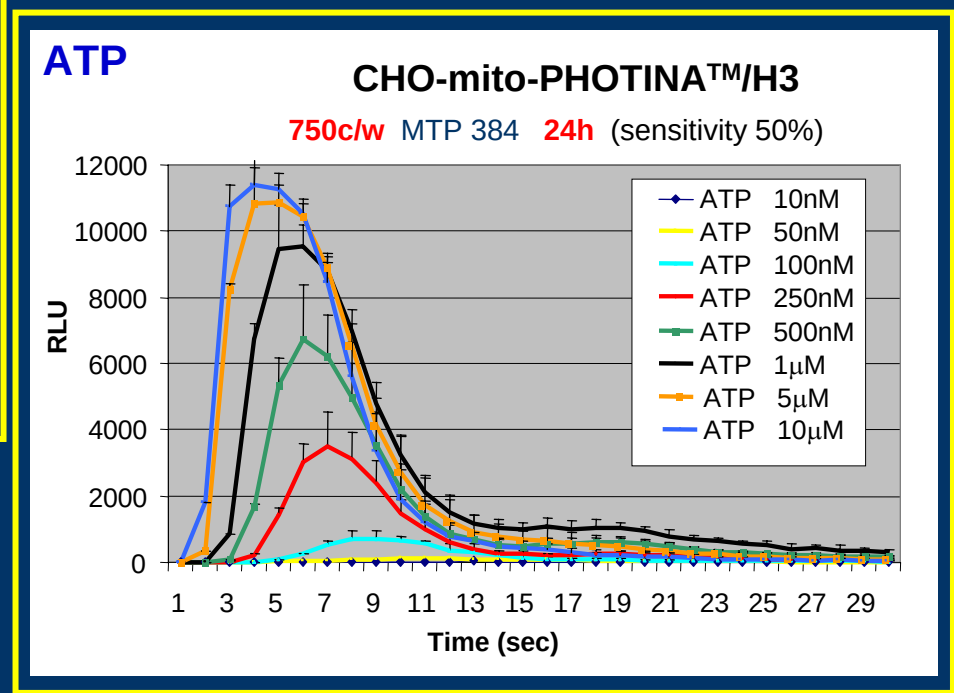
Characterisation of Photina™: CCD Camera

Histamine receptor 3, H3

agonist (IMETIT) dose-response



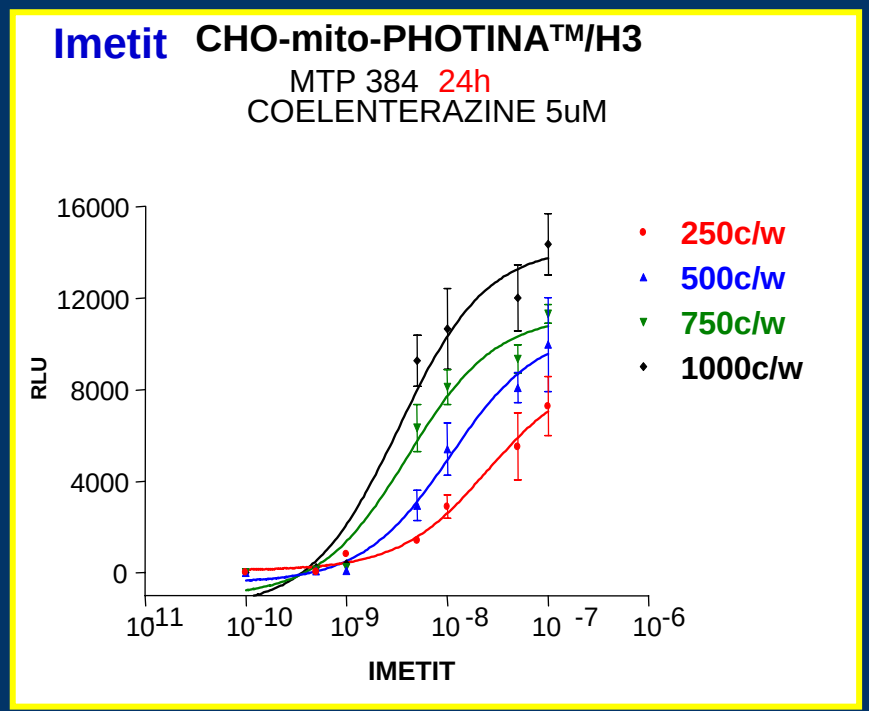
ATP dose-response

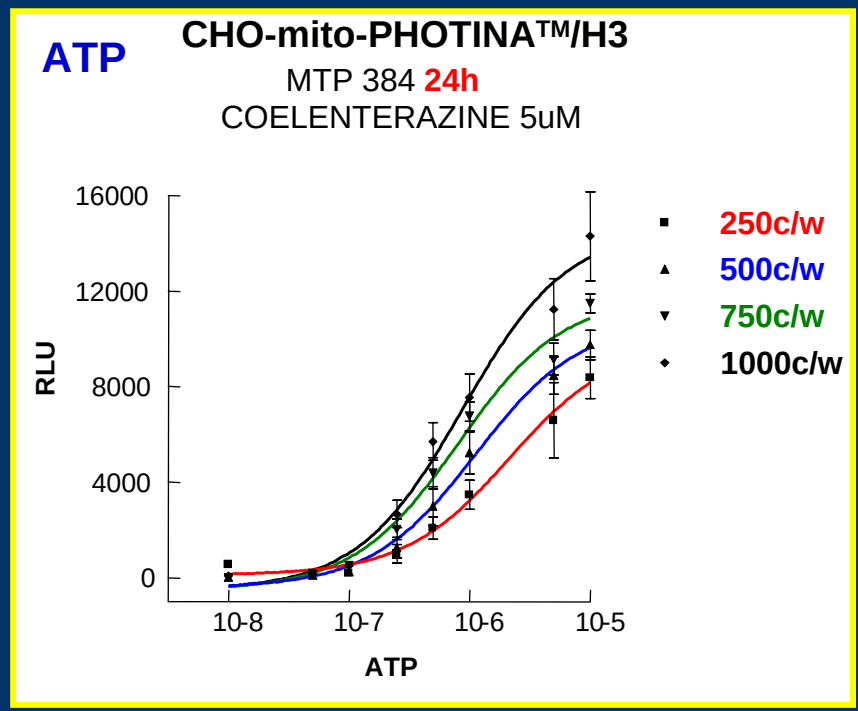


- 384 MTP
- 750 cells/well, 24 hrs
- 5 µM coelenterazine
- 3 hrs incubation at 37°C
- 50% CCD Camera sensitivity

Characteristics and Characterisation of Photina™

Histamine receptor 3, H3





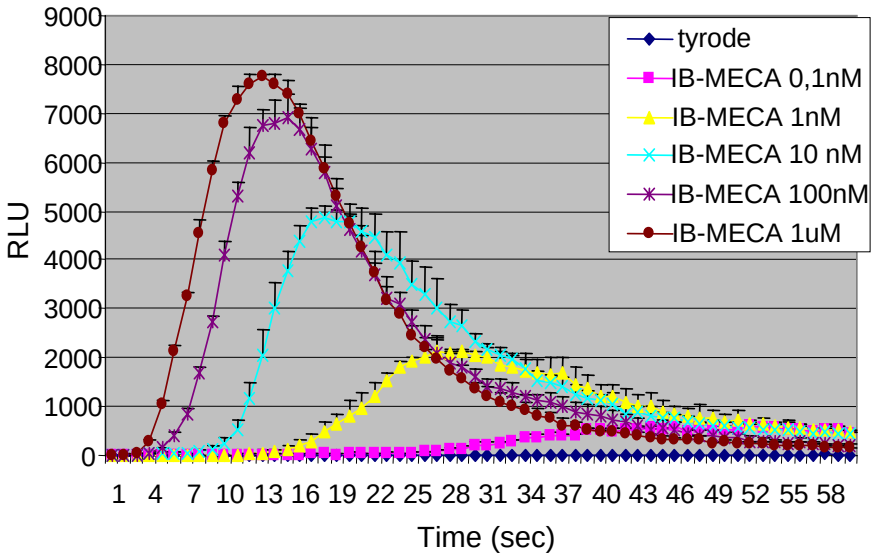
	EC_{50}
250 c/w	24 nM
500 c/w	11 nM
750 c/w	4 nM
1000 c/w	3,3 nM

	EC_{50}
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™

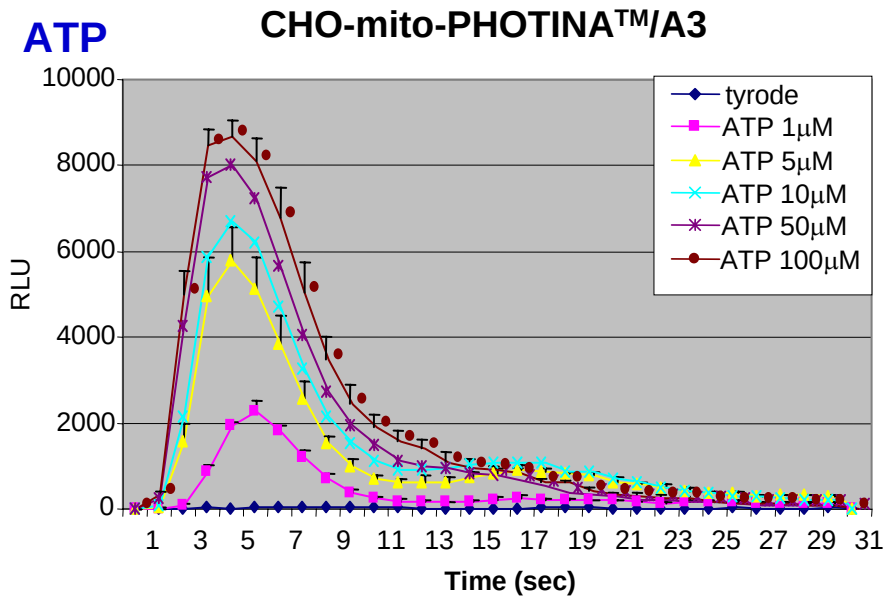
Adenosine-3 receptor, A3

agonist (IB-MECA) dose-response



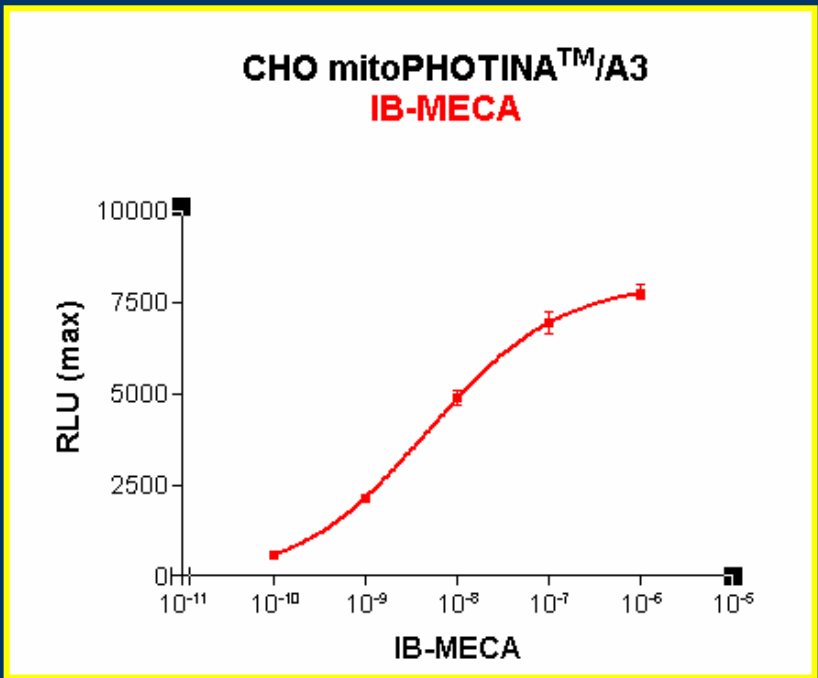
- 384 MTP
- 1000 cells/well, 24 hrs
- 5 μM coelenterazine
- 3 hrs incubation at 37°C
- 50% CCD Camera sensitivity

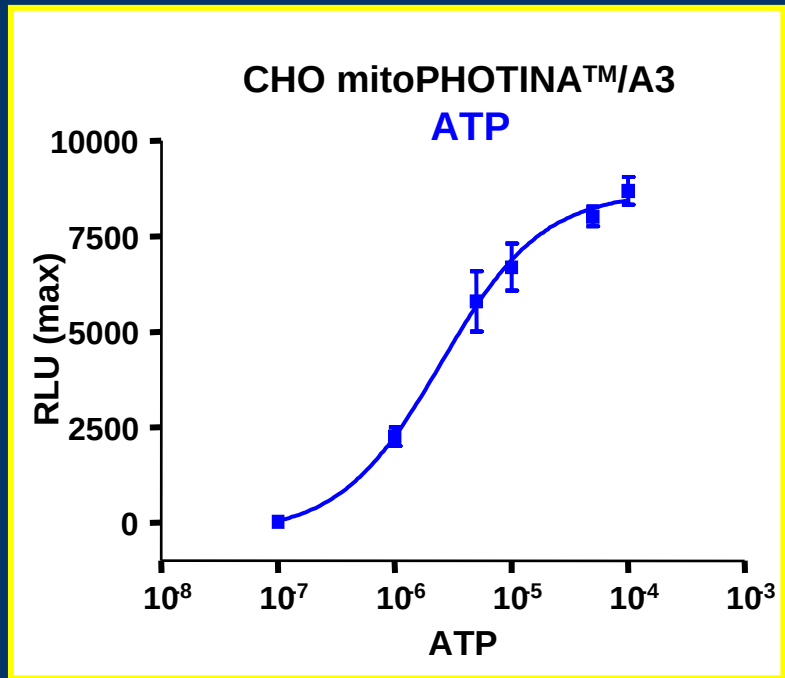
ATP dose-response



Characteristics and Characterisation of Photina™

Adenosine-3 receptor, A3





EC_{50}	R^2
4,7 nM	1

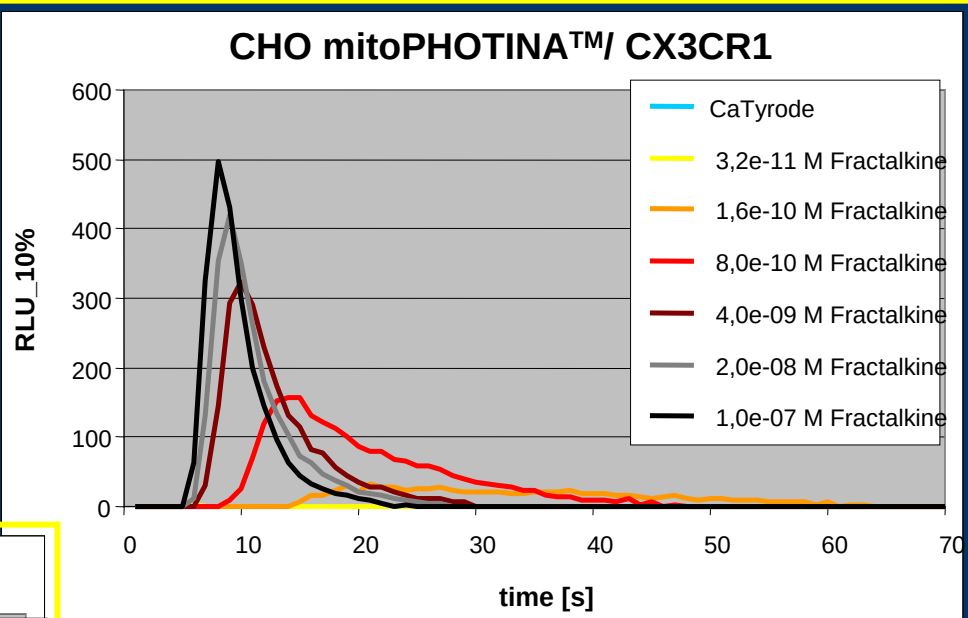
- 384 MTP
- 1000 cells/well, 24 hrs
- 5 μM coelenterazine
- 3 hrs incubation at 37°C
- 50% CCD Camera sensitivity

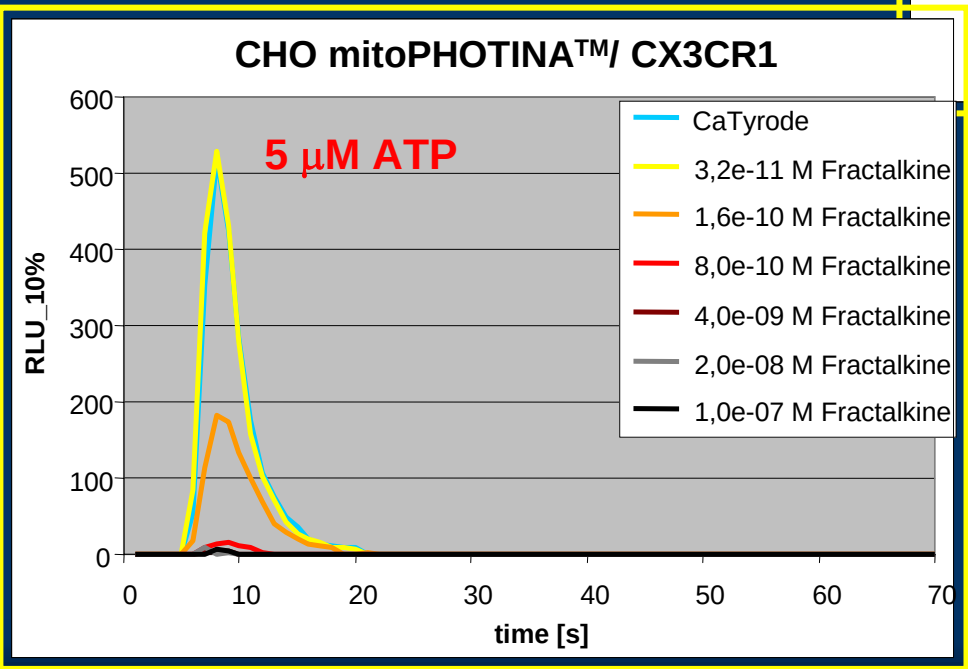
EC_{50}	R^2
2,5 μM	0,997

Characteristics and Characterisation of Photina™

1536 well format, CCD camera

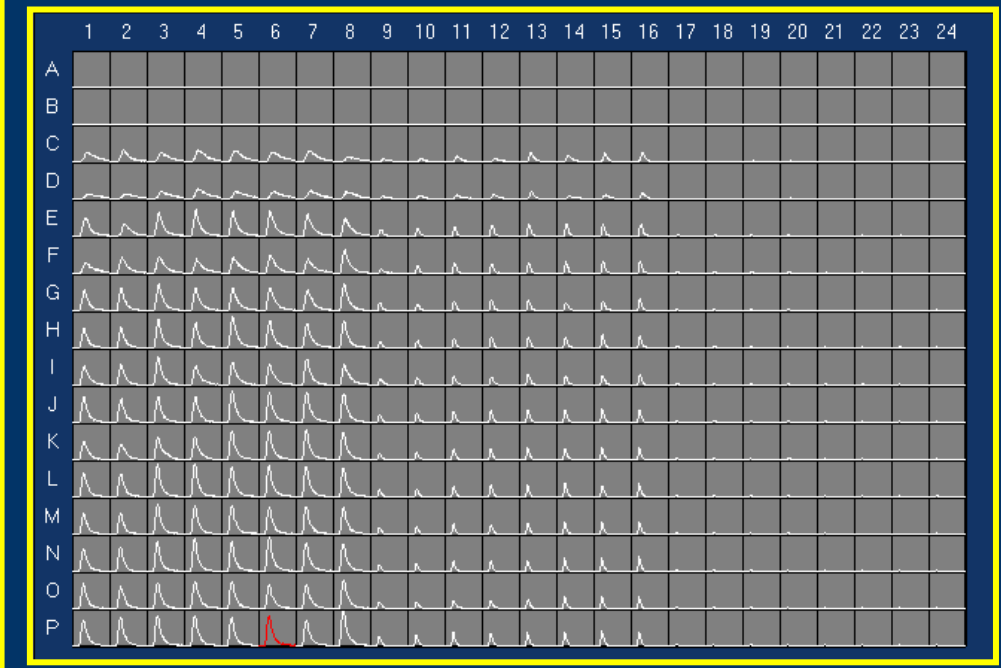
- 250 cells/well were plated in 1536 well plate 48 h before the experiment
- Cells were loaded with 2,5 μM coelenterazine for 2 h
- Luminescence was recorded with a CCD camera at 10% sensitivity



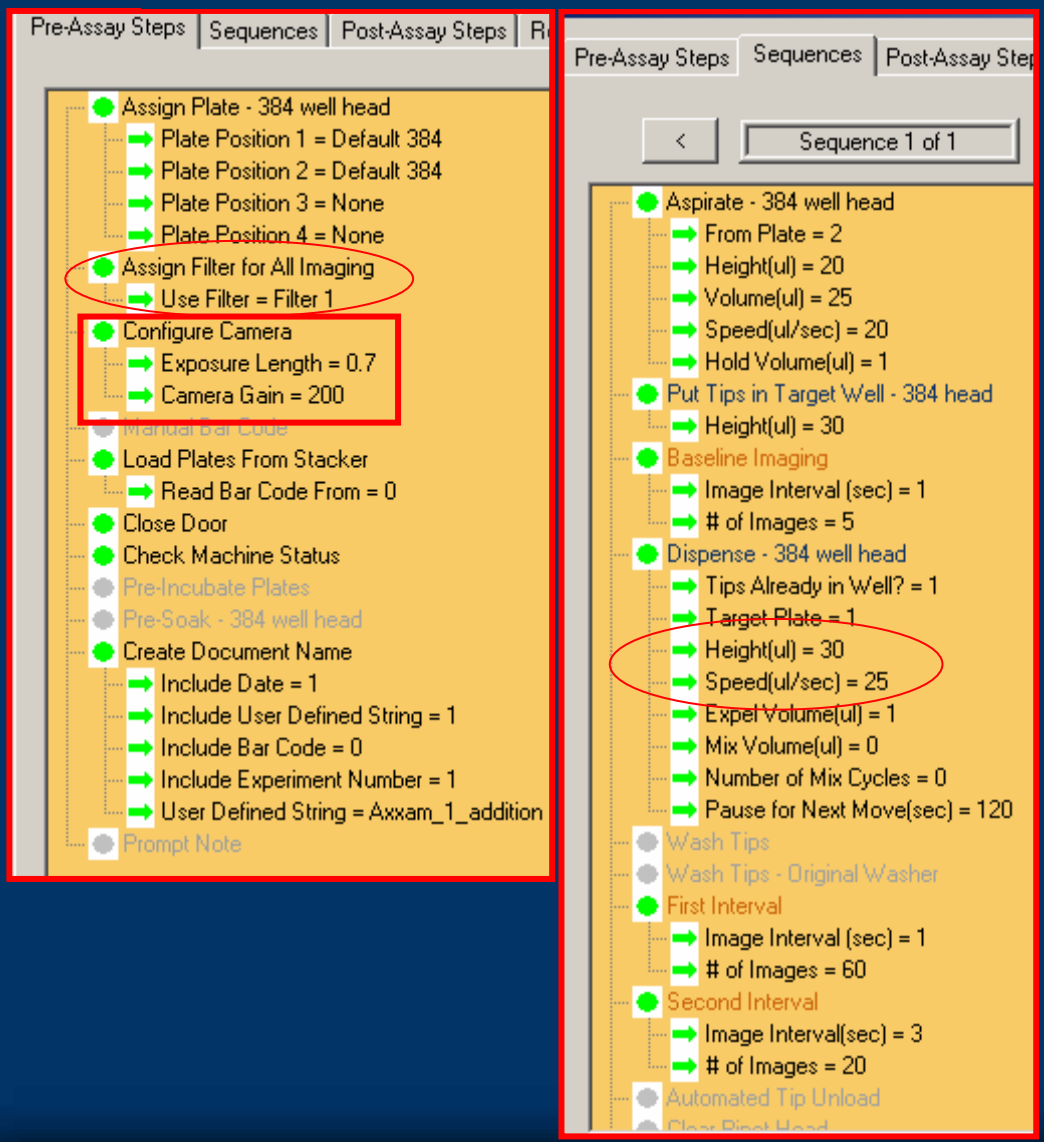


- Low number of cells/well required
- Low quantity of coelenterazine required
- Good pharmacology of endogenous and exogenous receptors

FLIPR3
&
Luminescence



FLIPR3 Settings for Photina™ Assays

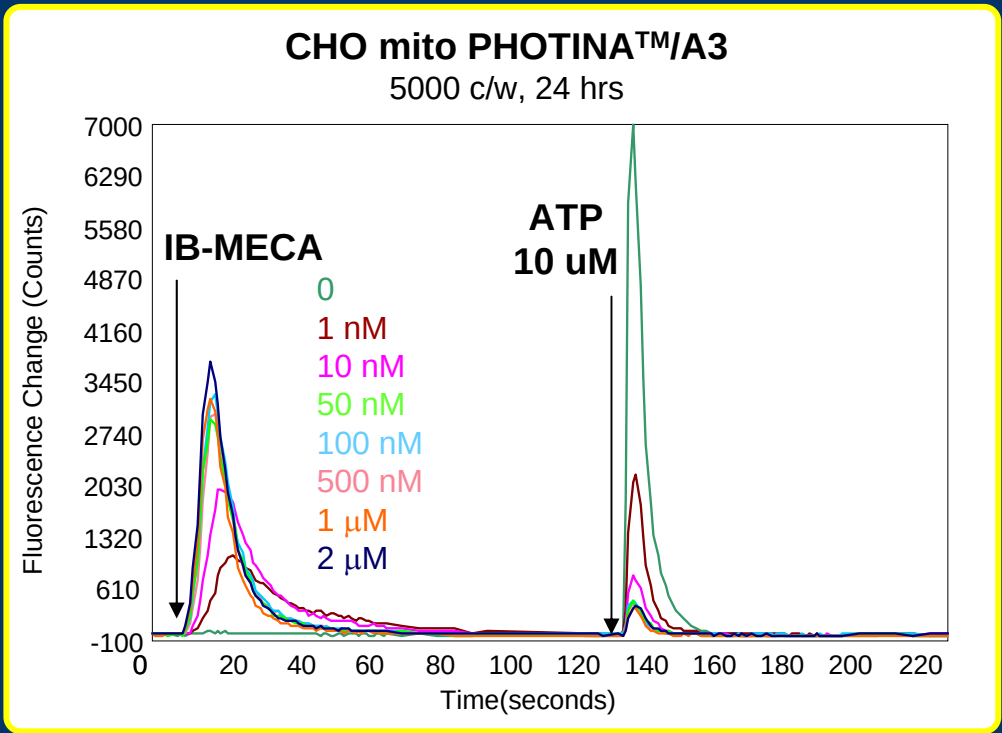


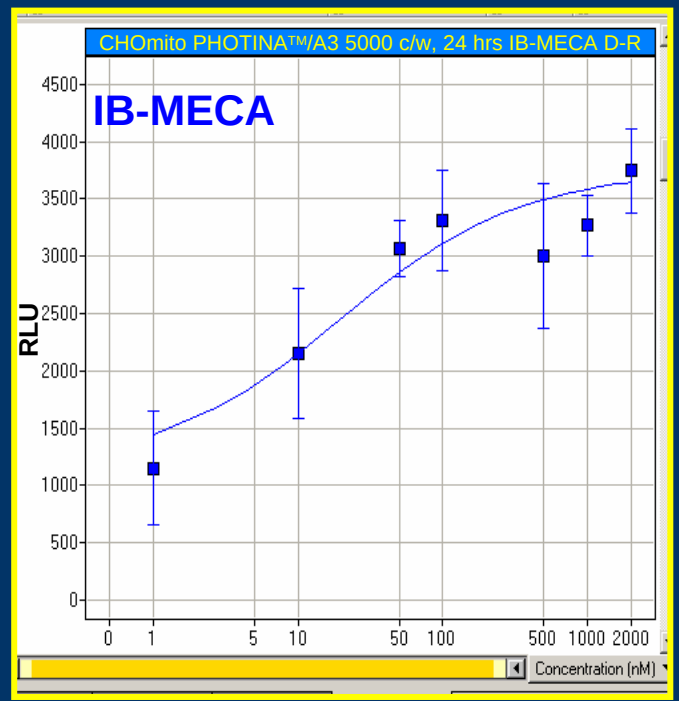
Standard protocol for Photina™ detection

- 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



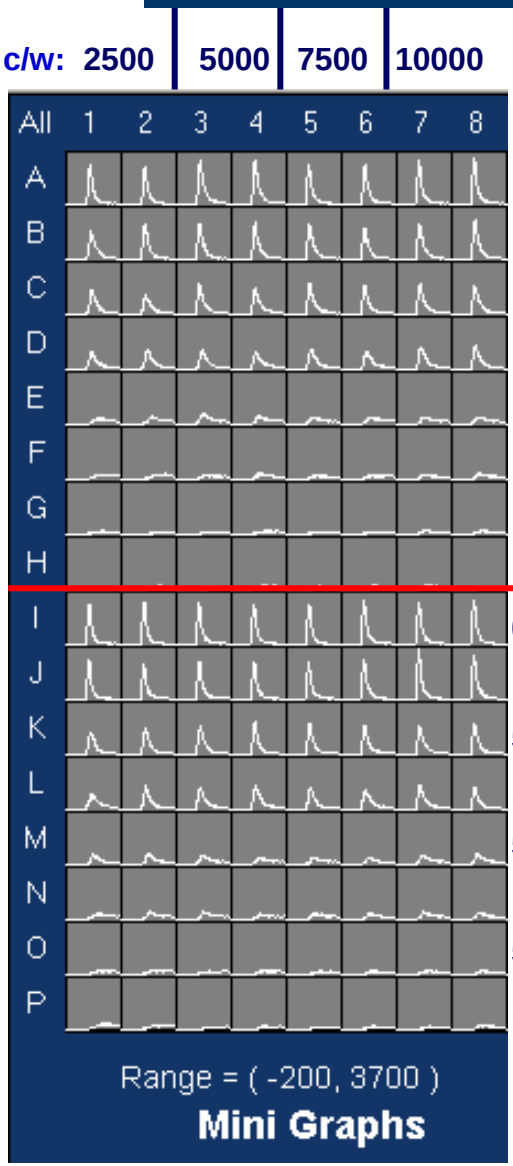


- 384 MTP
- 24 hrs
- 5 µM coelenterazine
- 4 hrs incubation at 37°C

	EC_{50}	R^2
5000 c/w	19 nM	0,886

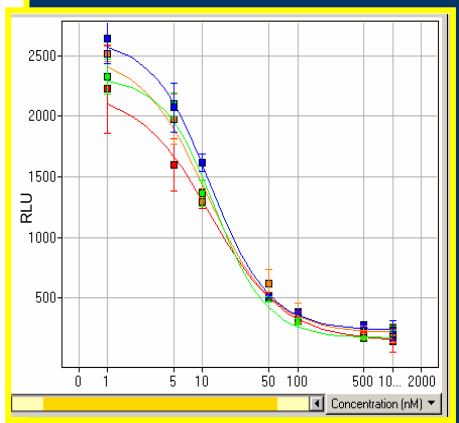
Adenosine Receptor 3, A3

Antagonist (MRS 1220) dose-inhibition



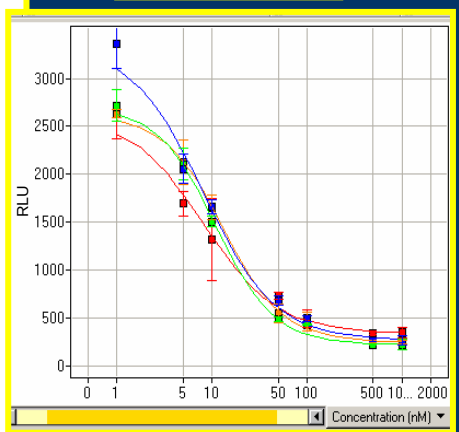
50 nM IB-MECA in the presence of increasing concentration of antagonist

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



	IC_{50}	R^2
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

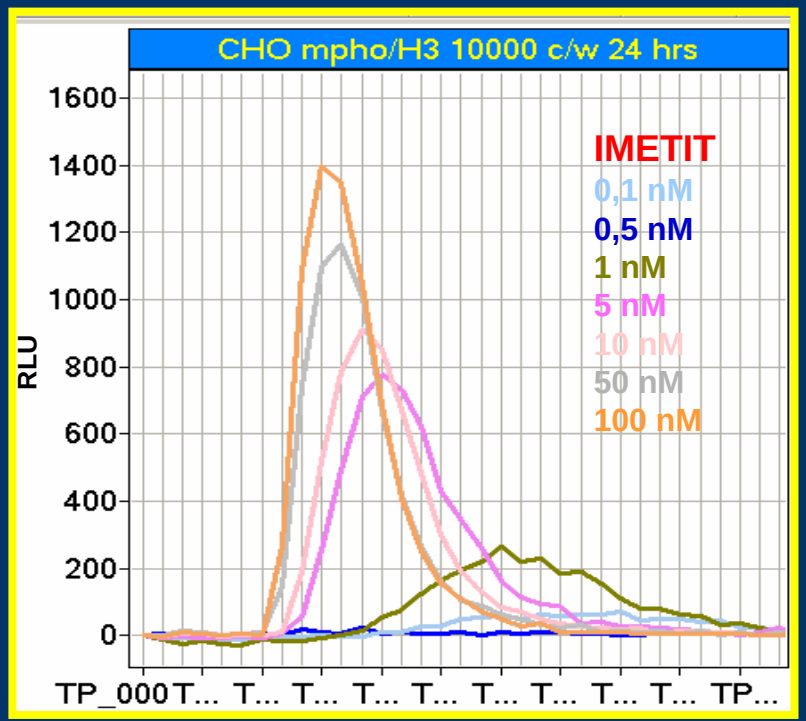
2500 c/w
5000 c/w
7500 c/w
10000 c/w

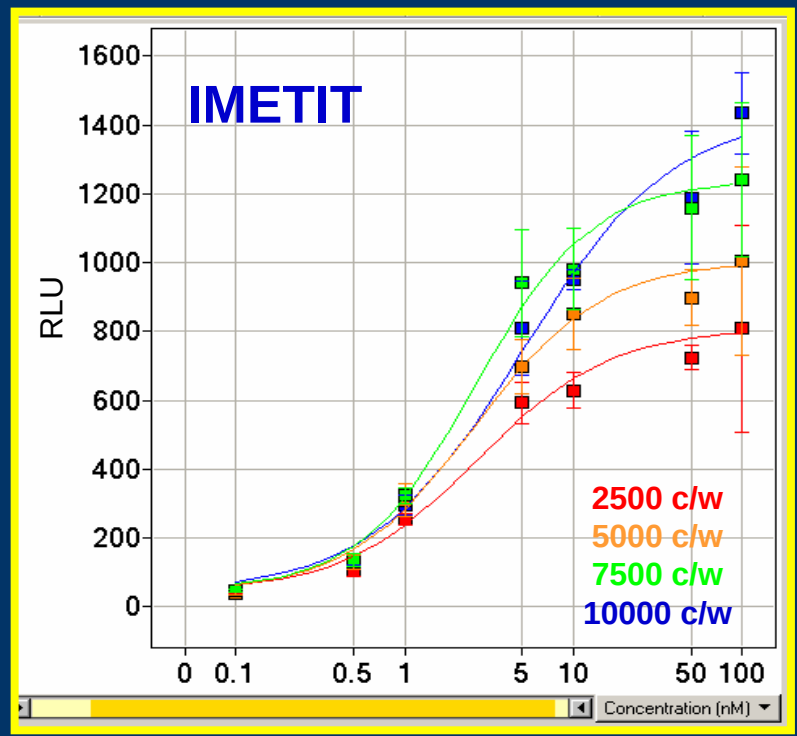


	IC_{50}	R^2
,2500 c/w	8 nM	0,985
5,000 c/w	13 nM	0,998
7,500 c/w	12 nM	0,997
10,000 c/w	8 nM	0,987

Histamine Receptor 3, H3

Agonist (IMETIT) dose-response



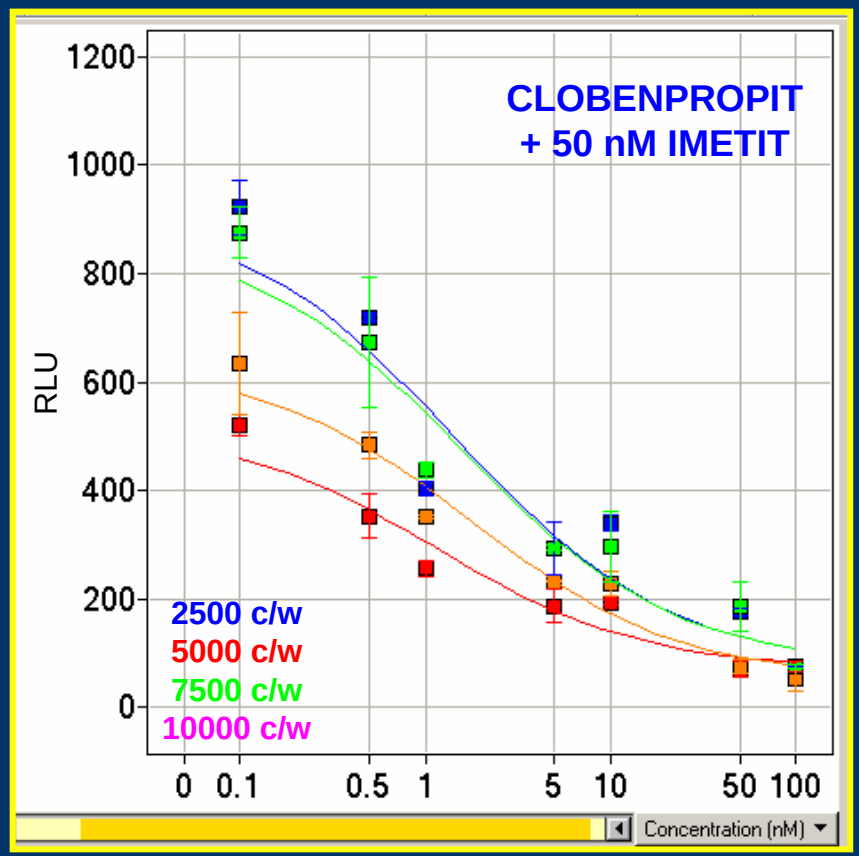


- 384 MTP
- 24 hrs
- 5 μM coelenterazine
- 4 hrs incubation at 37°C

	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

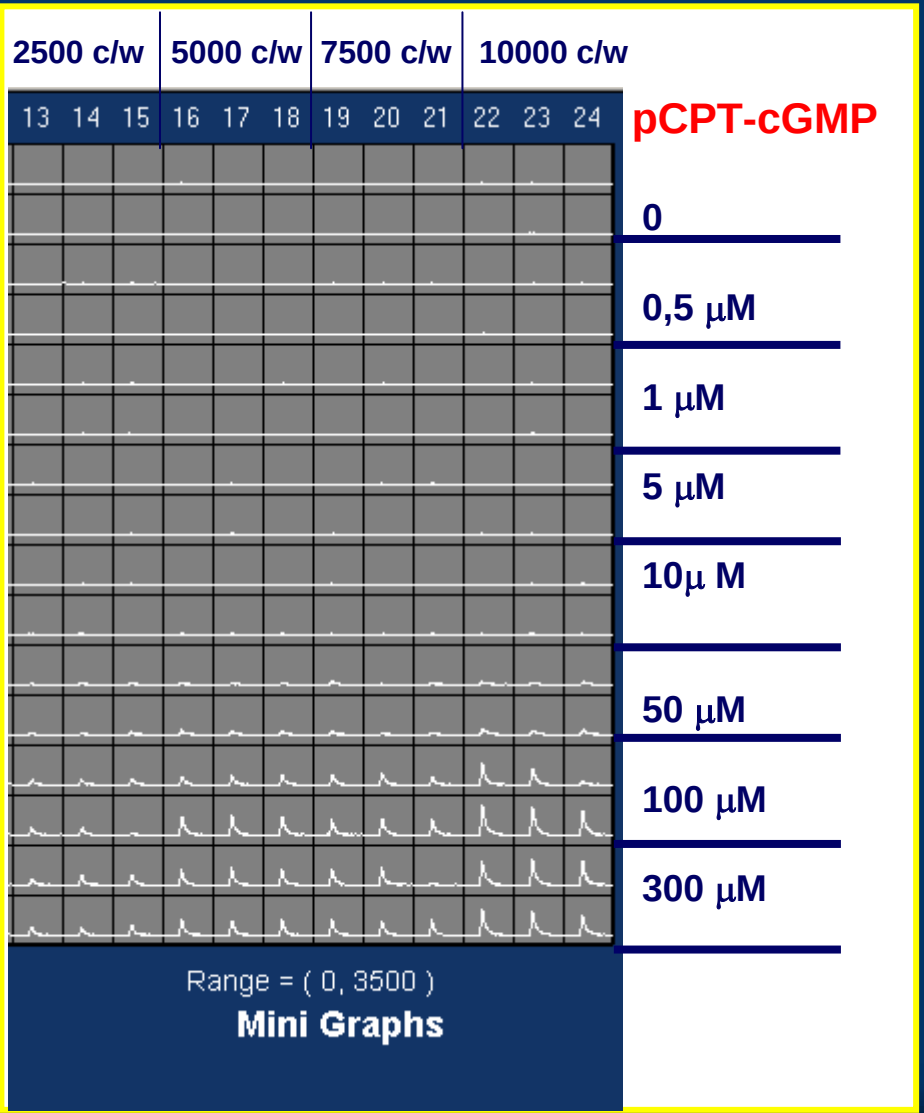


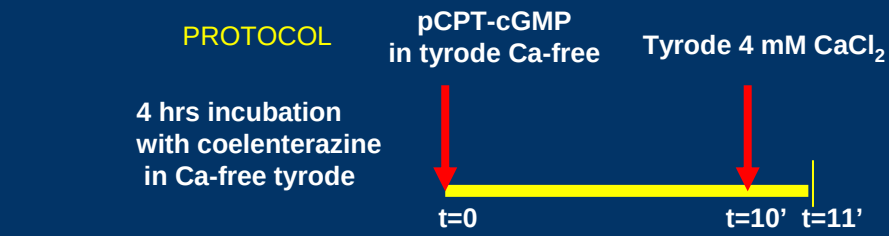
- 384 MTP
- 24 hrs
- 5 μM coelenterazine
- 4 hrs incubation at 37°C

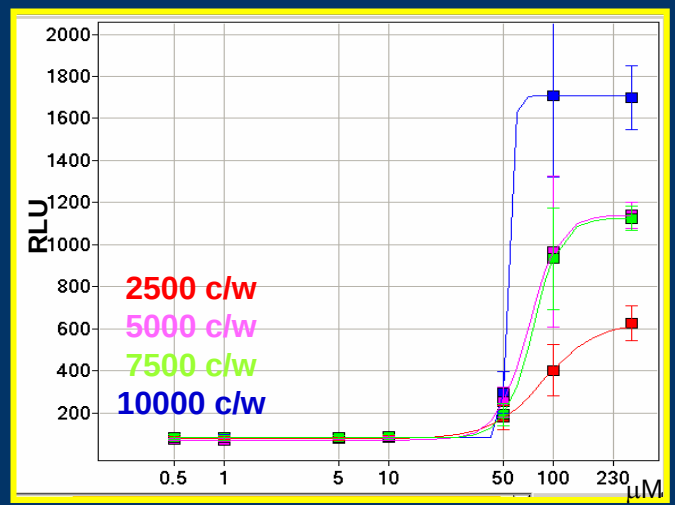
	IC_{50}	R^2
2,500 c/w	1,1 nM	0,937
5,000 c/w	1,7 nM	0,961
7,500 c/w	1,5 nM	0,939
10,000 c/w	1,4 nM	0,905

Cyclic Nucleotide-gated (CNG) Channel

Agonist (pCPT-cGMP) dose-response



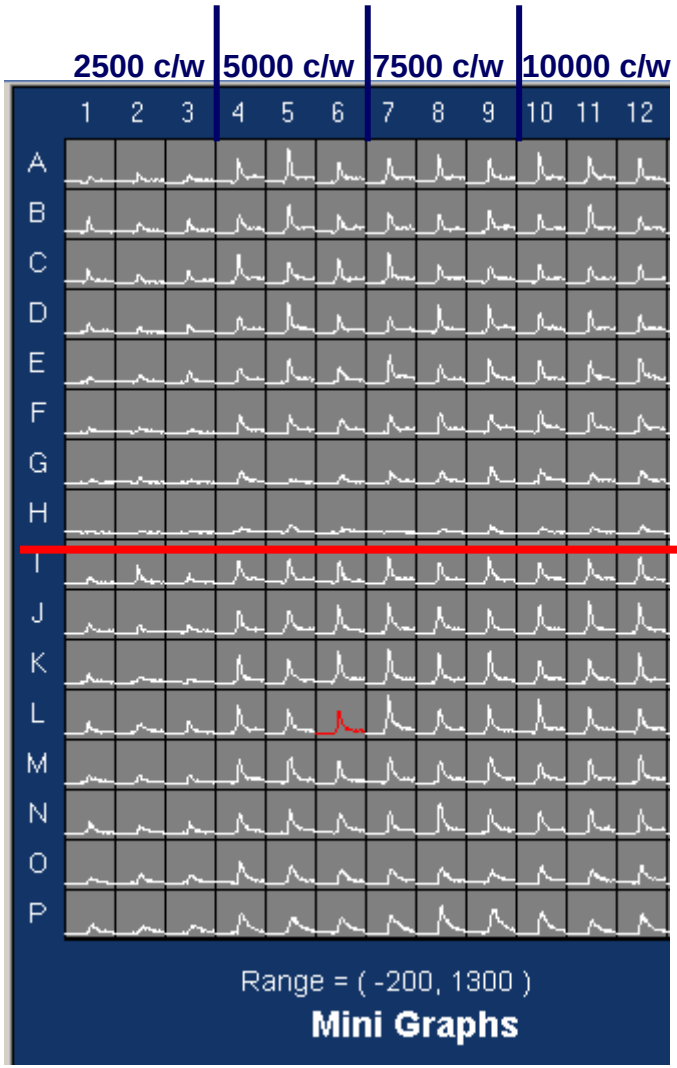




	EC_{50}	R^2
2,500 c/w	86 µM	0,999
5,000 c/w	70 µM	1
7,500 c/w	75 µM	1
10,000 c/w	53 µM	1

Vanilloid Receptor 1 (VR1)

Antagonist (CAPSAZEPINE) dose-inhibition



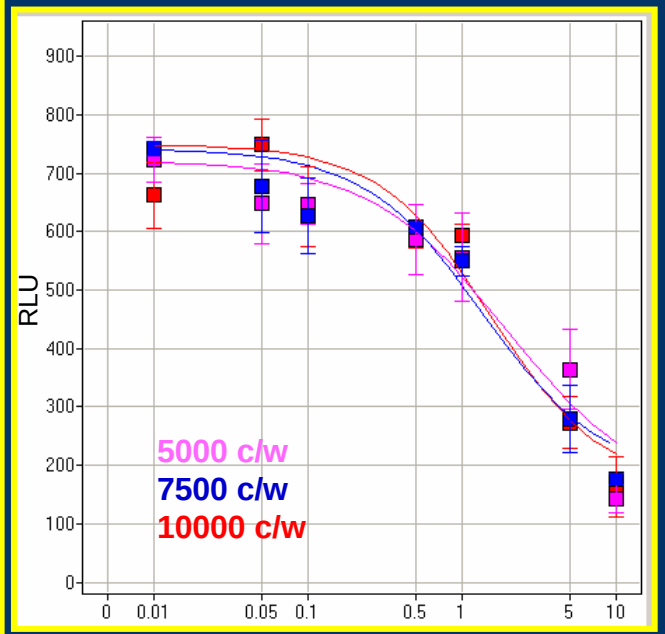
CZ

- 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)

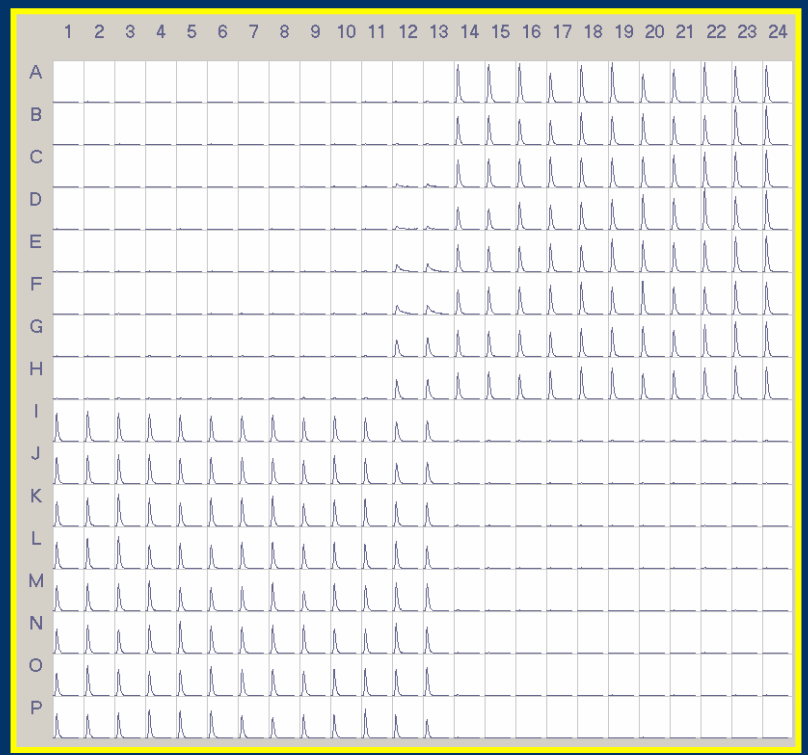
- 0
- 0,01 µM
- 0,05 µM
- 0,1 µM
- 0,5 µM
- 1 µM
- 5 µM
- 10 µM

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

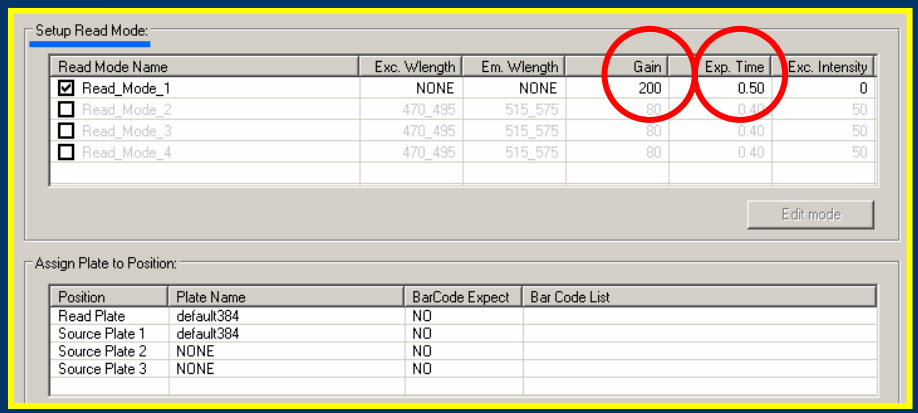


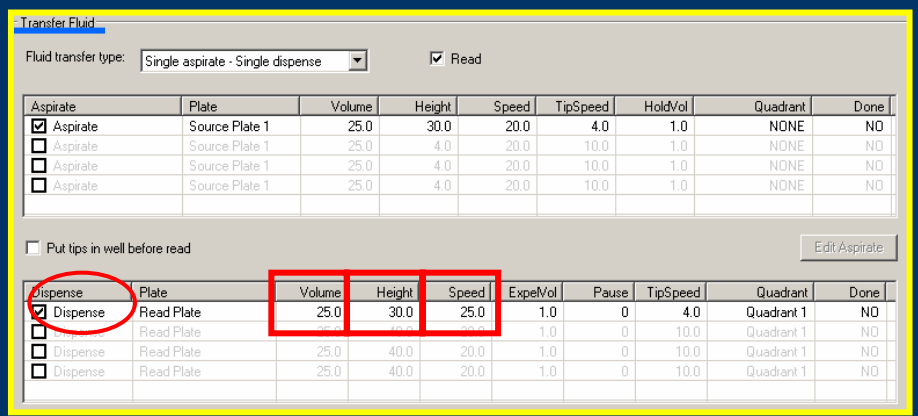
	IC_{50}	R^2
5,000 c/w	1,9 µM	0,921
7,500 c/w	1,5 µM	0,918
10,000 c/w	1,3 µM	0,945

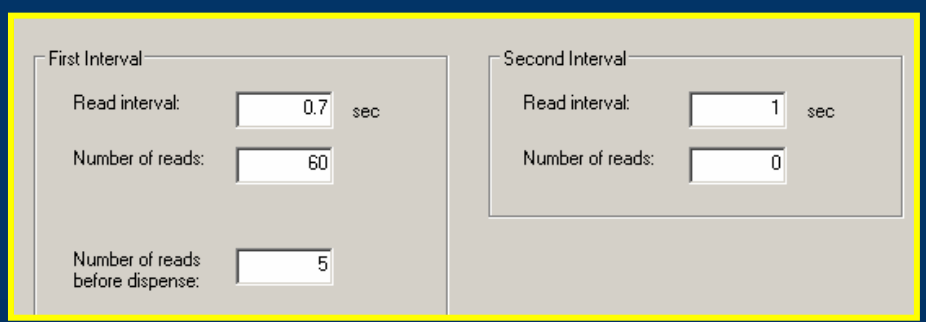
FLIPR TETRA
&
Luminescence



FLIPR TETRA Settings for Photina™ Assays





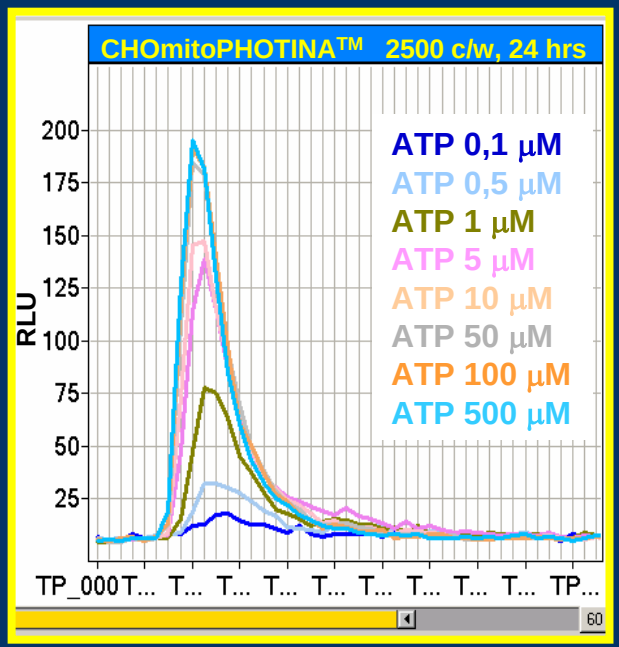


Standard protocol for Photina™ detection

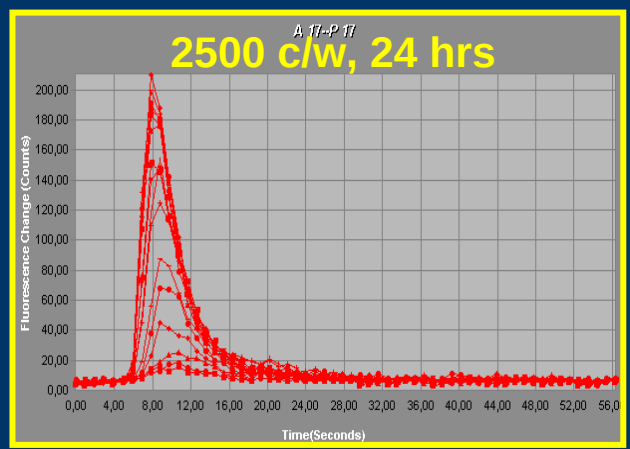
- 384 white wall clear bottom plates
- cell plating 24 hrs before the experiment
- medium removal
- addition of tyrode + coelenterazine 25µl/well
- incubation 4 hrs at 37°C
- experiment run at FLIPR TETRA: compounds (2X) injection in tyrode buffer (25µl/well)

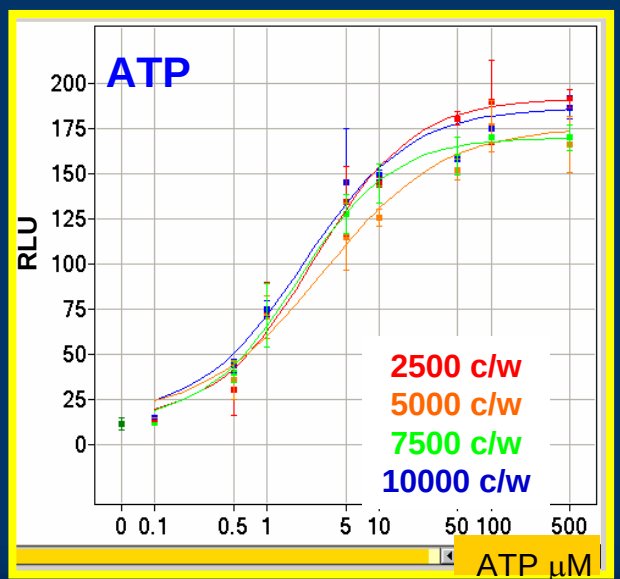
Endogenous P2Y Receptors

ATP response

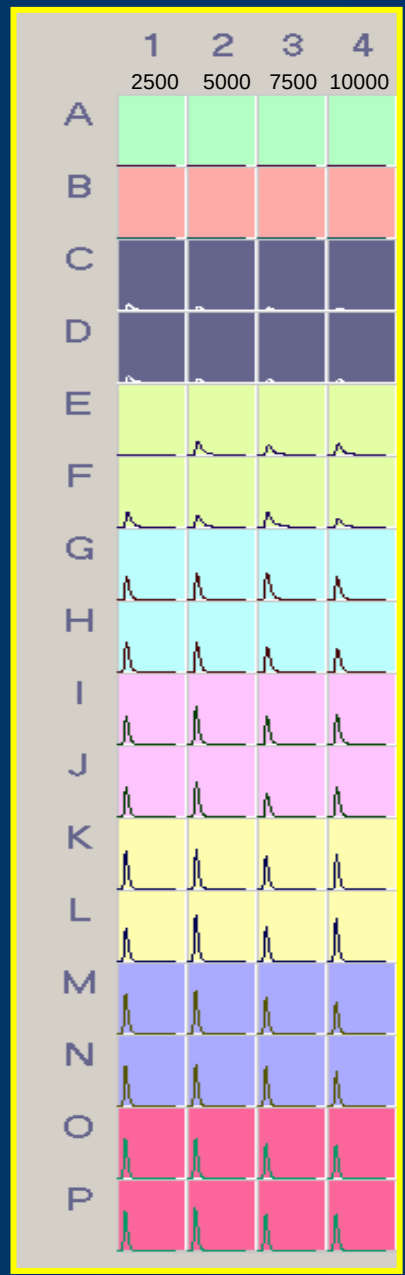


- 384 MTP
- 24 hrs
- 5 µM coelenterazine
- 4 hrs incubation at 37°C



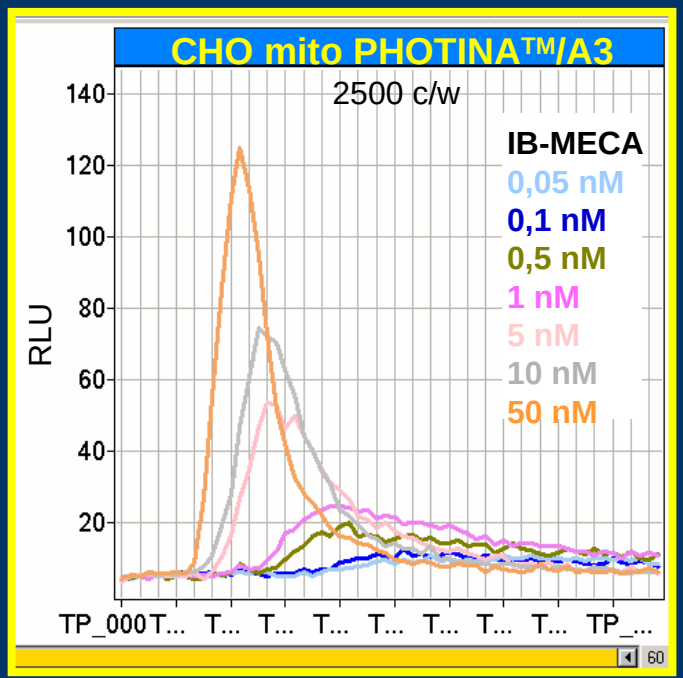


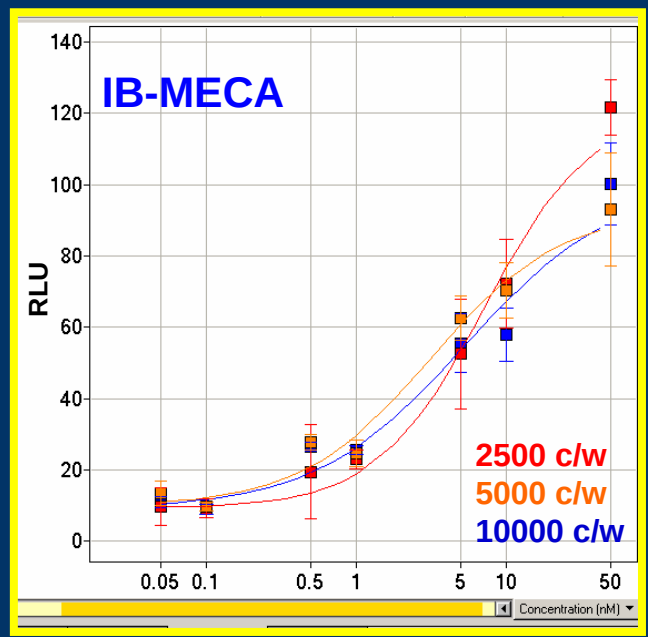
	EC_{50}	R^2
2,500 c/w	2,6 µM	0,988
5,000 c/w	3,1 µM	0,979
7,500 c/w	1,8 µM	0,995
10,000 c/w	2,1 µM	0,974

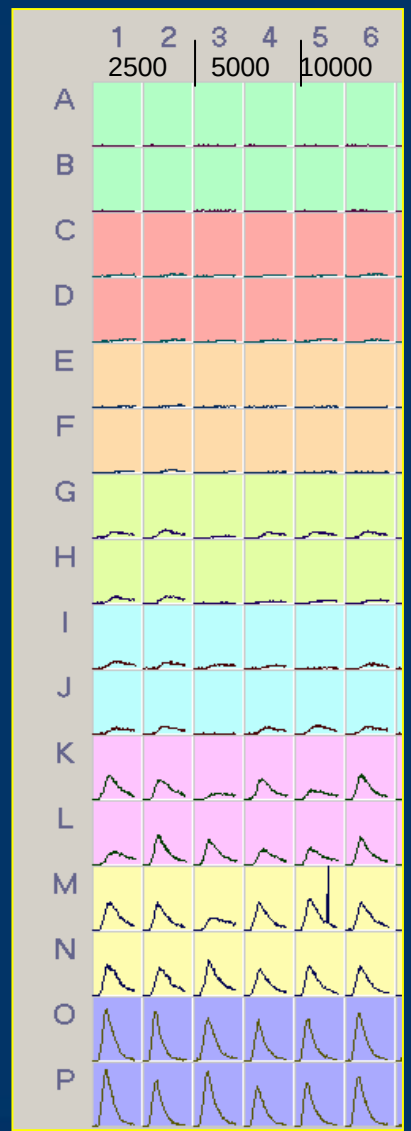


Adenosine Receptor 3, A3

Agonist (IB-MECA) dose-response





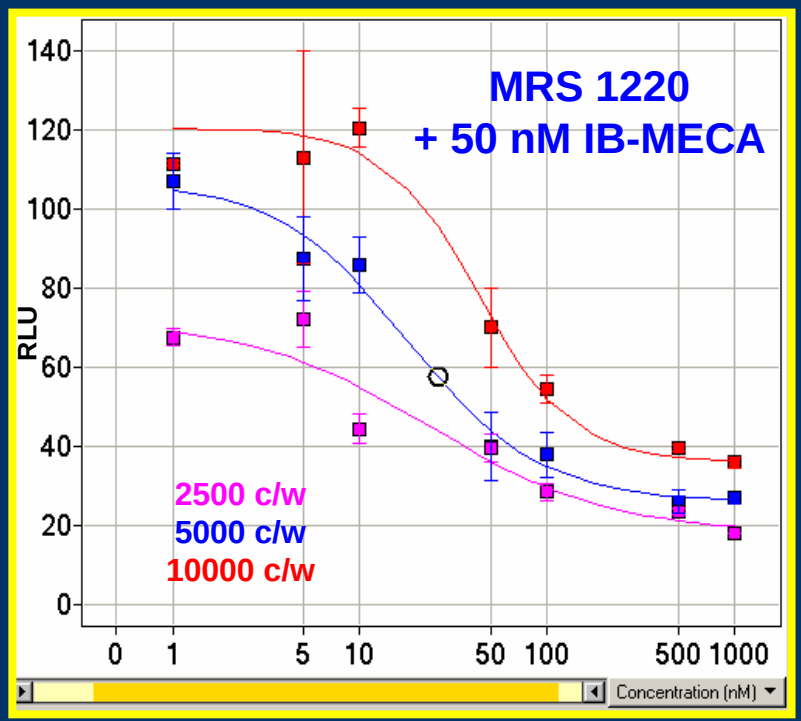


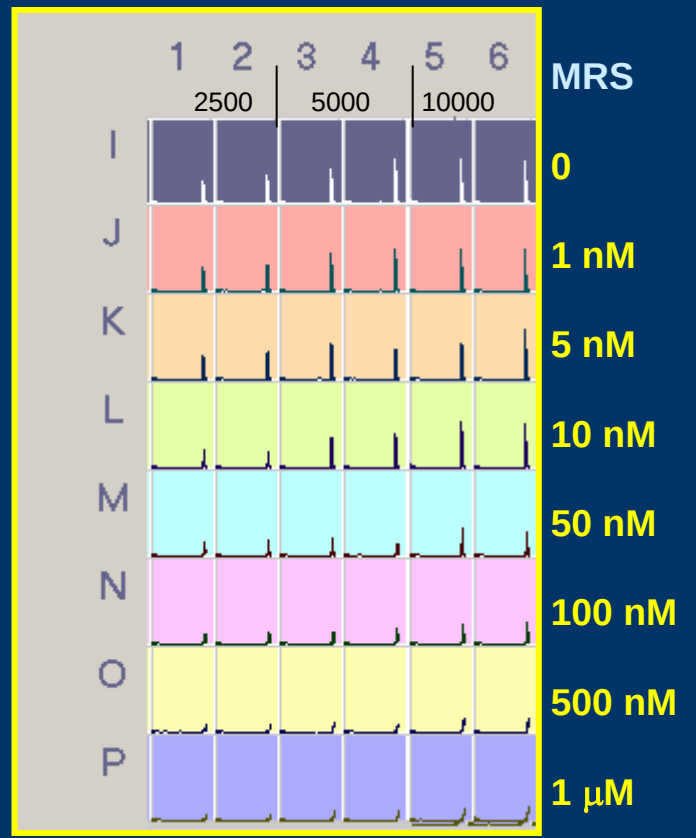
- 384 MTP
- 24 hrs
- 5 μM coelenterazine
- 4 hrs incubation at 37°C

	EC_{50}	R^2
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



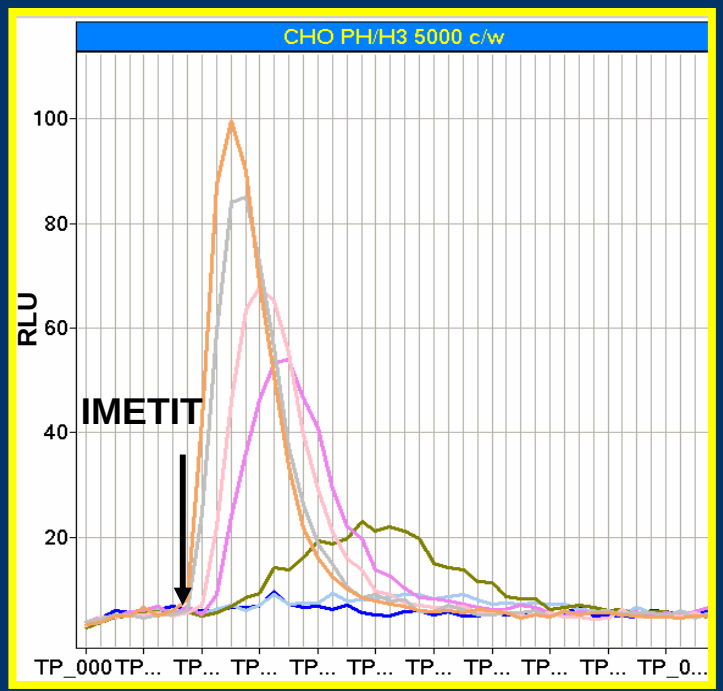


	IC_{50}	R^2
2,500 c/w	23 nM	0,907
5,000 c/w	18 nM	0,986
10,000 c/w	43 nM	0,979

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

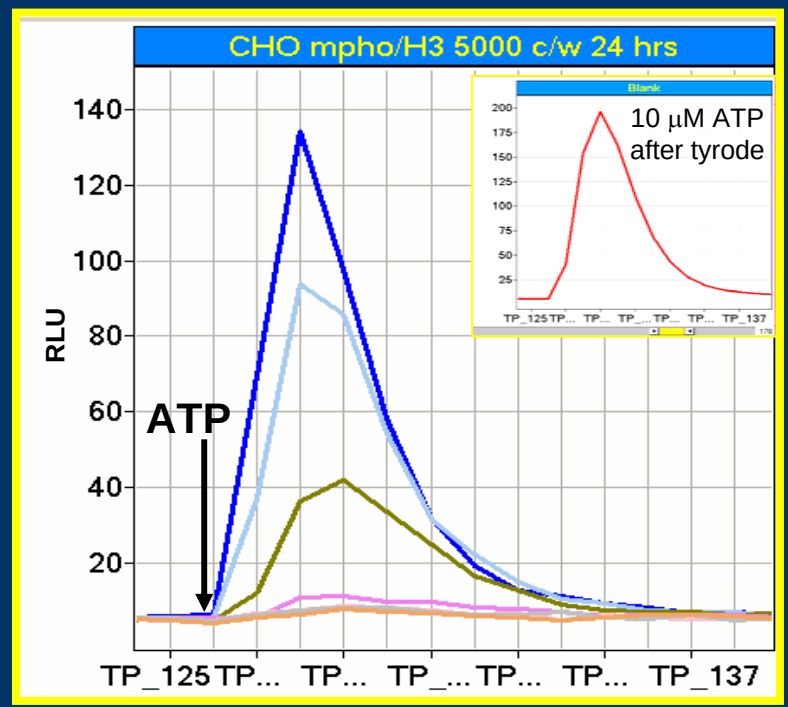
Histamine Receptor 3, H3

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 μM ATP has been injected 10' after a D-R to IMETIT

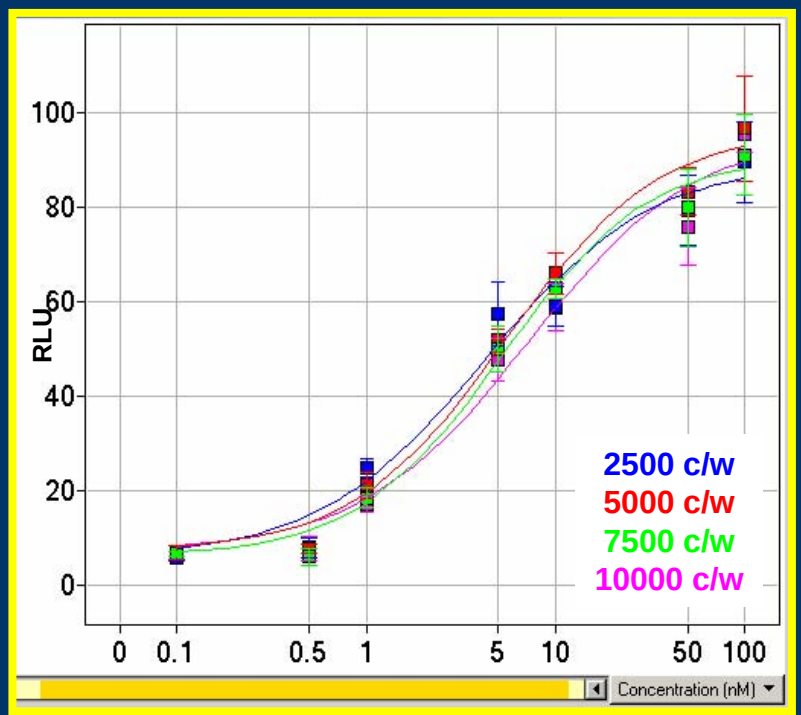


- 384 MTP
- 24 hrs
- 5 μM coelenterazine
- 4 hrs incubation at 37°C

Histamine Receptor 3, H3

Agonist (IMETIT) pharmacology

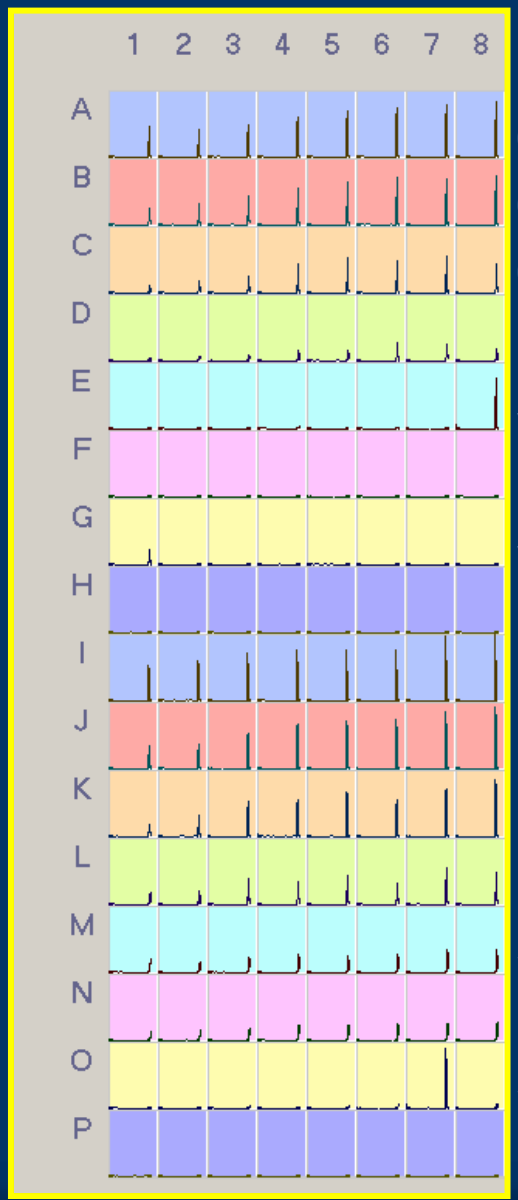
agonist (IMETIT) dose-response



	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

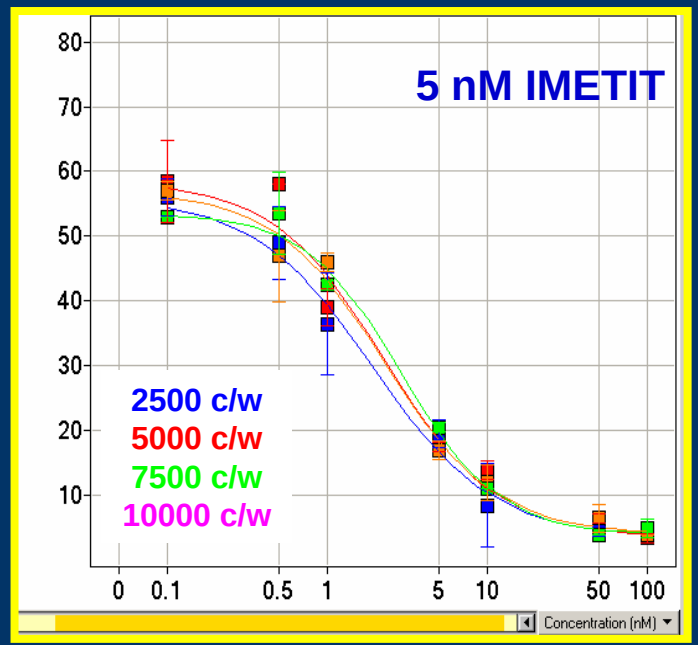
Antagonist (CLOBENPROPIT) pharmacology



5 nM IMETIT

50 nM IMETIT

antagonist (CLOBENPROPIT) dose-inhibition

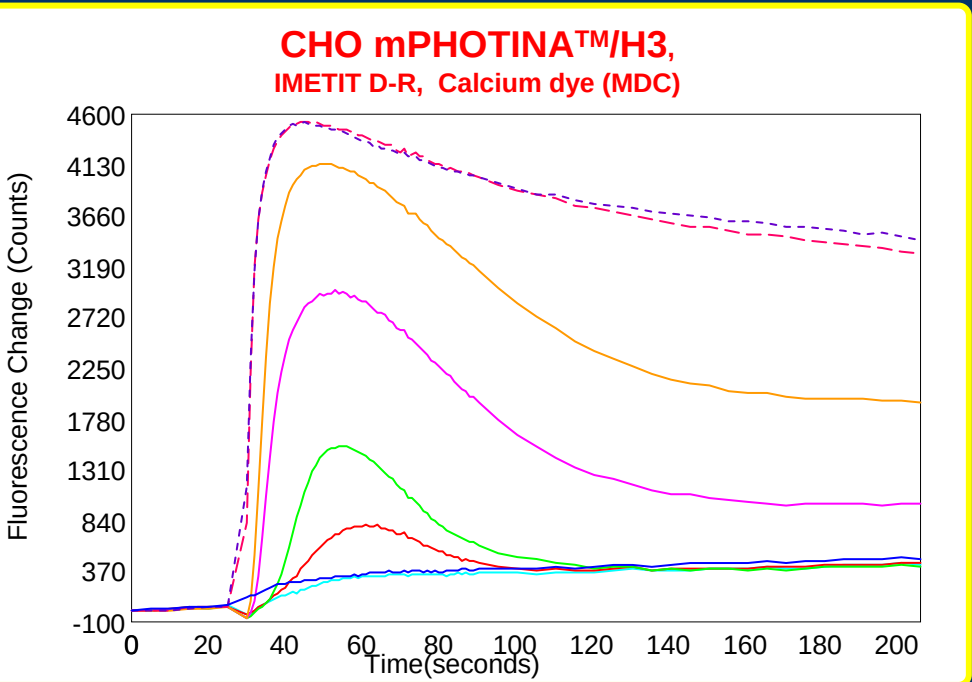


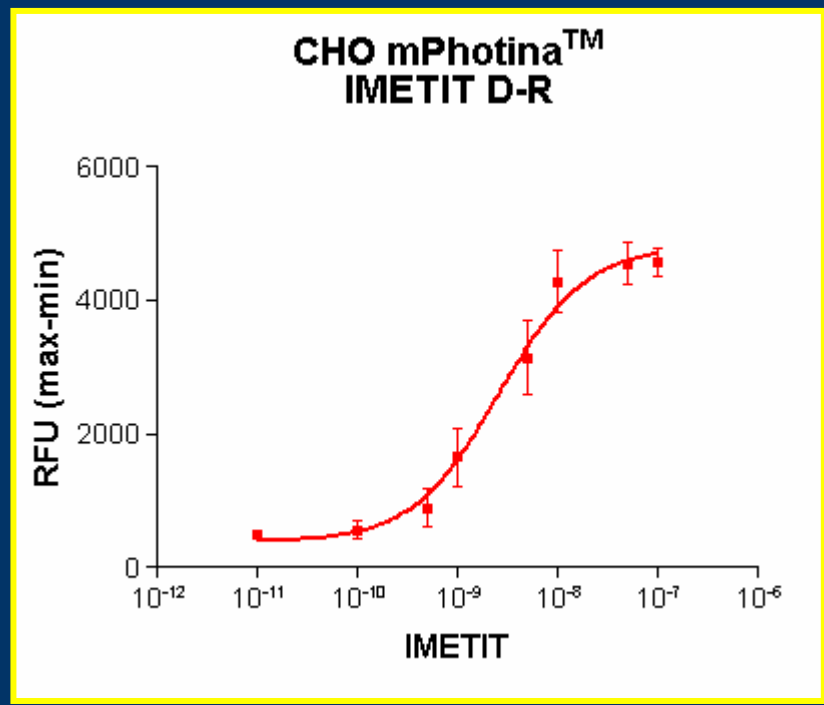
	IC_{50}	R^2
2,500 c/w	2,3 nM	0,974
5,000 c/w	2,3 nM	0,992
7,500 c/w	2,9 nM	0,993
10,000 c/w	1,9 nM	0,991

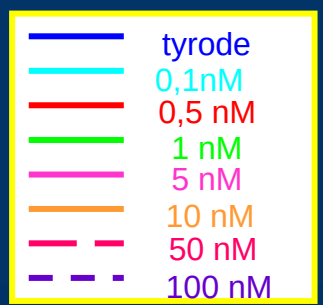
FLIPR_FLUORESCENCE

Histamine receptor 3, H3

IMETIT D-R





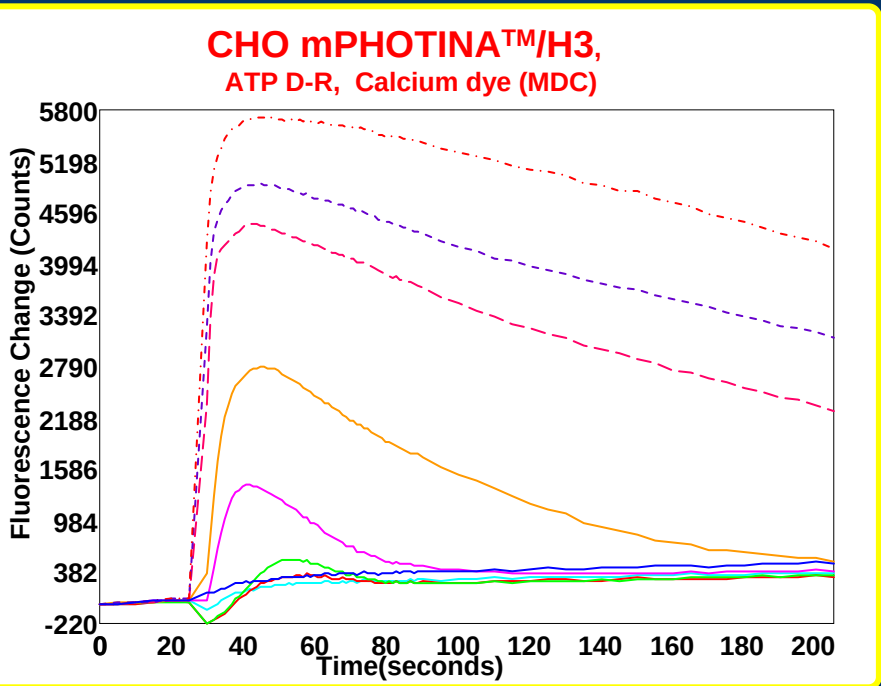


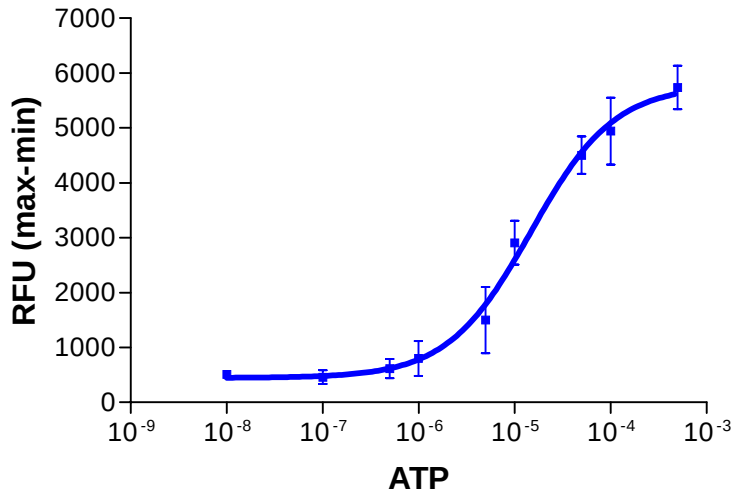
	EC_{50}	R^2
10,000 c/w	2,6 nM	0,989

FLIPR_FLUORESCENCE

Histamine receptor 3, H3

ATP D-R





	EC_{50}	R^2
10,000 c/w	14 μM	0,993

EC_{50} 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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