New tools offered or developed for a better understanding of the complexity of 7TM pharmacology

Dr. Xavier Leroy, DD, Actelion Pharmaceuticals Ltd, Switzerland

Novel Applications and Cutting Edge Technologies for GPCR Screening and Profiling
Chesterford Research Park
GPCR Conference 22nd/23rd September 2009

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Agenda

- Application of Phage-display Technology to GPCRs in Native Conformation
- Transduction Pathways:
  - Development of New Luciferase Reporter Gene Assays for the Characterization of GPCR Constitutive Activity and/or for the Characterization of the Signaling of Orphan GPCRs
  - Multiplexing Calcium and Beta-arrestin read outs on FLIPR Tetra – Application to Screening and Profiling
- Application of the FRET (SNAP-CLIP) Technology for the Characterization of GPCR Homo- and Hetero-dimers
- Application of the DiscoveRx Beta-arrestin Cell-based GPCR Assay for Receptor De-orphanisation

Application of Phage-display Technology to GPCRs in Native Conformation

Phage-Display / GPCRs – Reverse Pharmacology

- Developed by Smith 1985 (Smith G.P., Science 228, 1985).
- A bacteriophage is a virus that infects only bacteria and is not infectious for human.
- Contains genetic material surrounded by a coat protein.
- Phage genome causes infected bacteria to make more phages.
- Standard tool: filamentous, non lytic phages M13 or fd (ss DNA).
- New genetic material is inserted into the phage genome.
- Infected E.coli processes the new gene and exposes the new protein on the phage surface.
- Based on the fusion of a protein or antibody with the pIII minor coat protein of M13.

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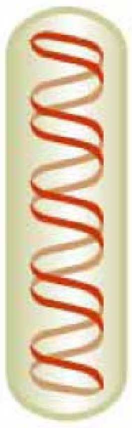
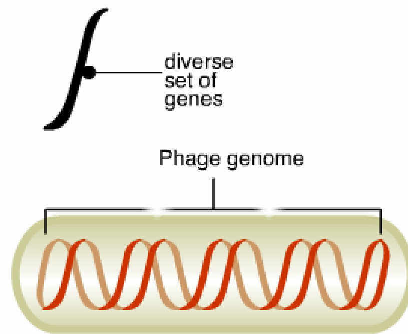
Phage-Display / GPCRs – Reverse Pharmacology

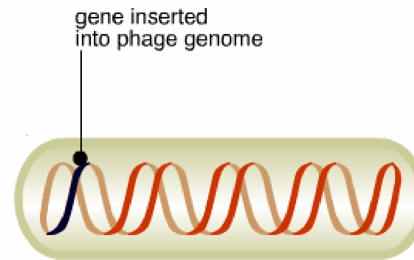
Phage Structure



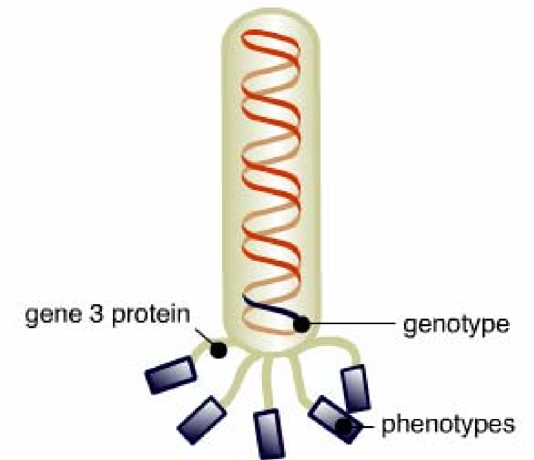
Inserting different genes into phage genome



Different phages carry different genes



Added gene expresses a protein, antibody or peptide on the phage structure

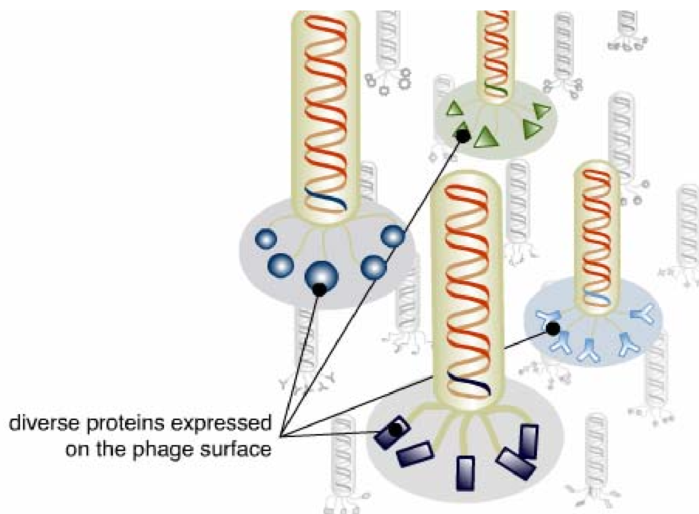


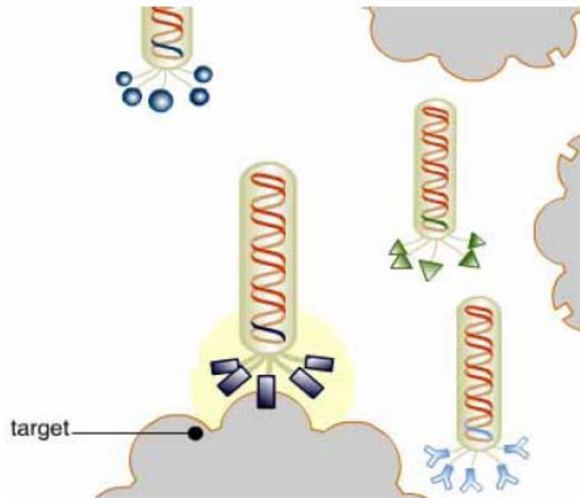
Phage-Display / GPCRs – Reverse Pharmacology

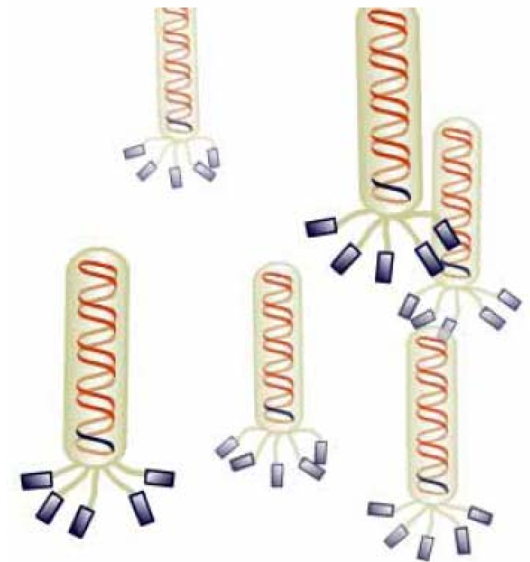
Diverse libraries can be generated

A selected library is exposed to an immobilized target (protein, disease causing molecule, receptor)

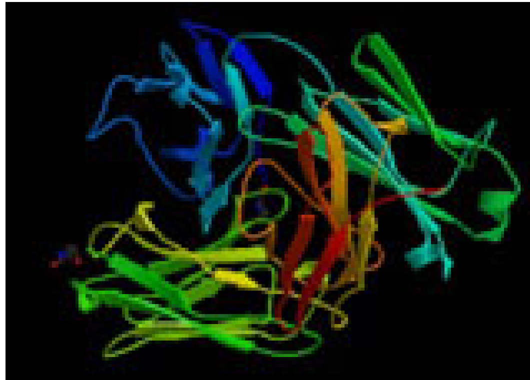
Purification, amplification, and isolation of a candidate gene (“biopanning”)



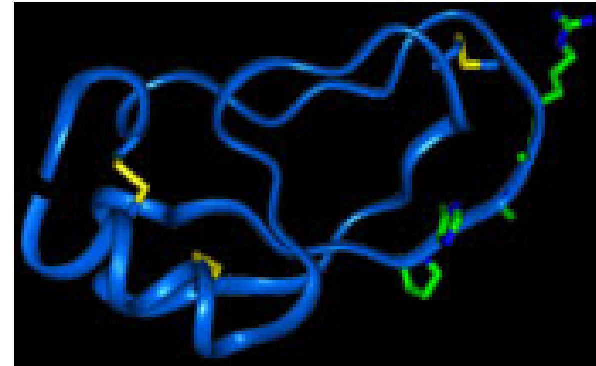




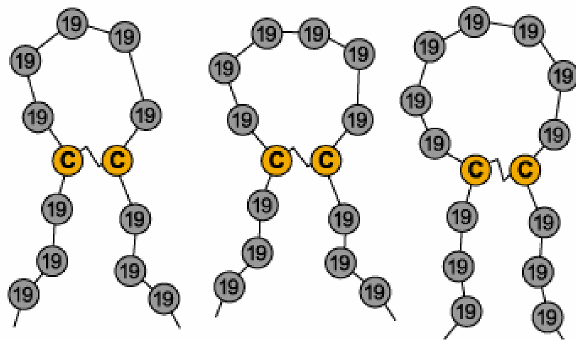
Phage-Display / GPCRs – Reverse Pharmacology



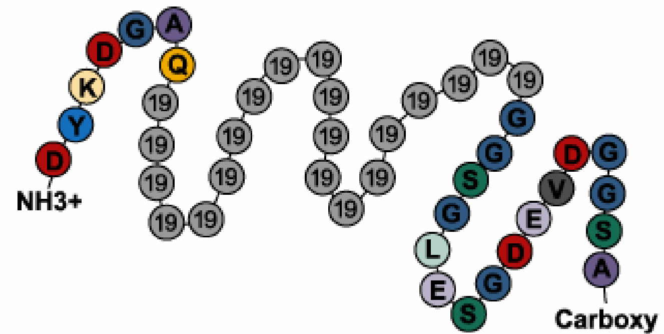
Antibody libraries



Protein libraries

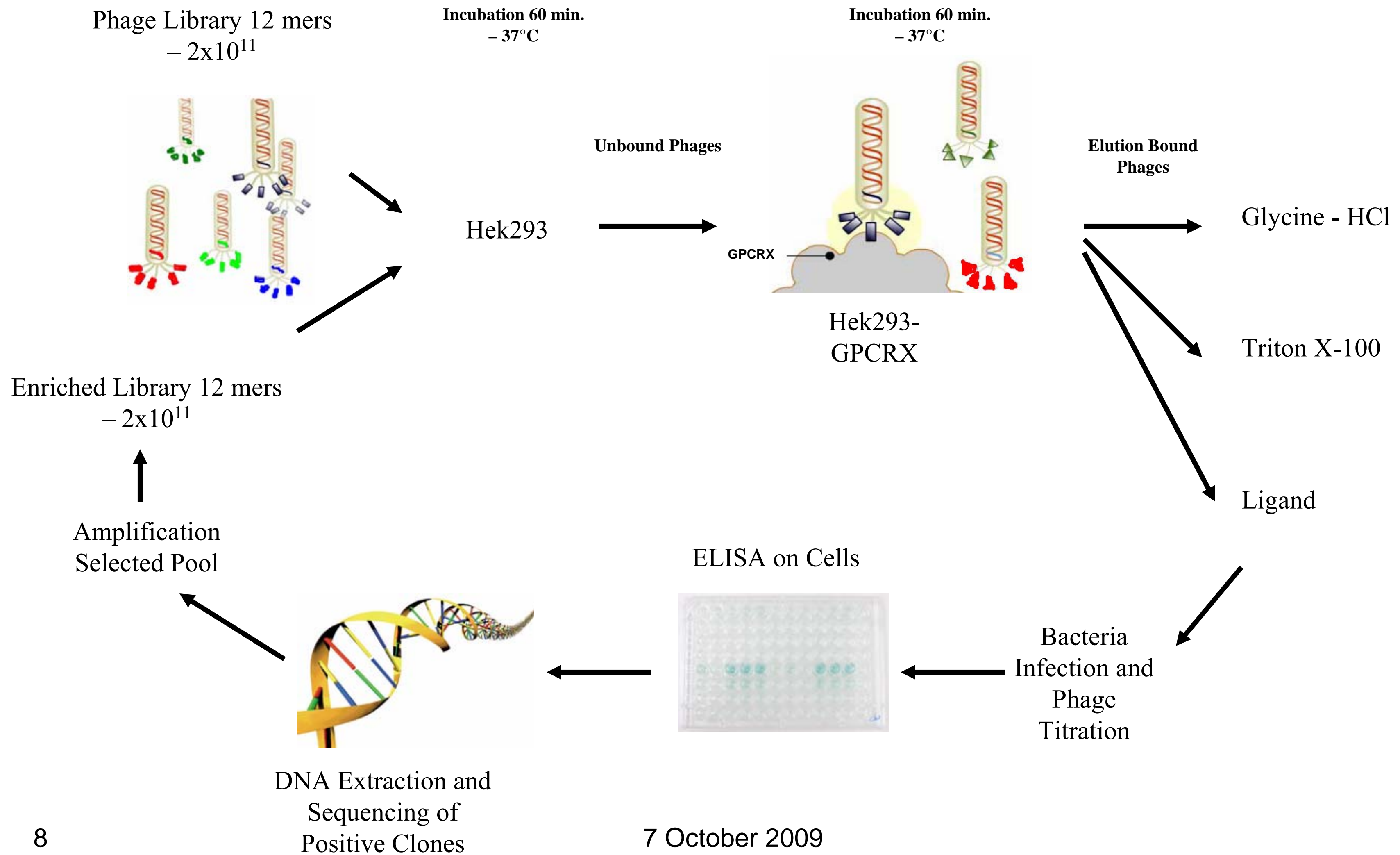


Cyclic Peptide libraries



Linear libraries

Phage-Display / GPCRs – Reverse Pharmacology

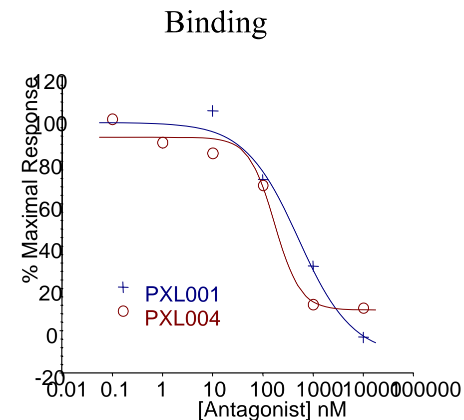


Phage-Display / GPCRs – Reverse Pharmacology

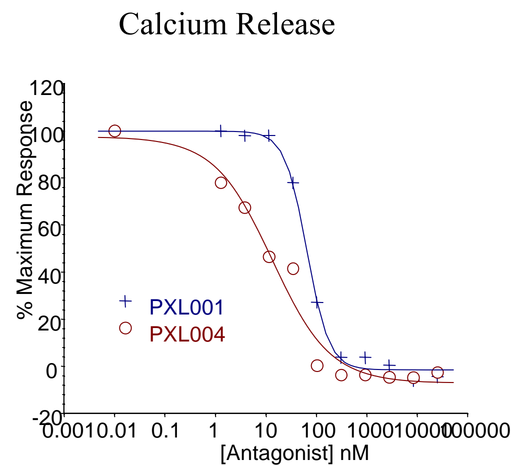
- Synthesis of deduced peptides.
- Evaluation on recombinant cell-lines in various assays (FLIPR, Binding, GTP-γ-S).
- Evaluation on primary cells (Migration, Activation).

Name	Binding	FLIPR	GTP-g-S
PXL602	7	3	80
PXL004	17	25	225
PXL606	26	28	143
PXL603	34	65	740
PXL713	97	159	N.T.
PXL704	157	113	N.T.
PXL001	160	69	361
PXL701	172	77	N.T.
PXL714	294	272	N.T.
PXL702	325	578	N.T.
PXL605	835	2980	N.T.
PXL604	1306	863	>10.000
PXL705	2225	2830	N.T.

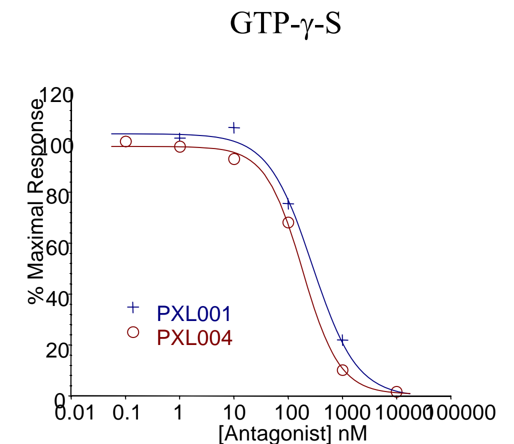
Binding



Calcium Release



GTP-γ-S



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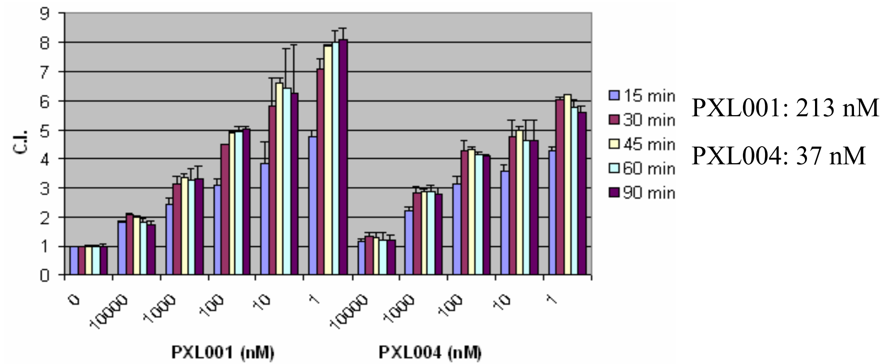
Phage-Display / GPCRs – Reverse Pharmacology

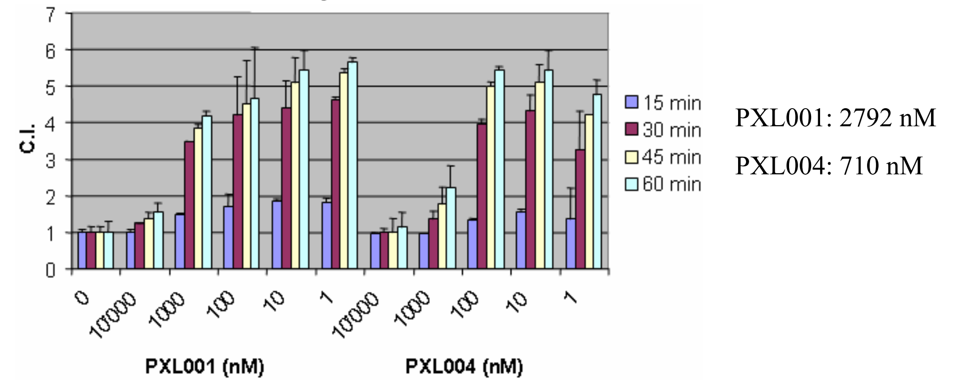
Inhibition of Human Neutrophil Migration



PXL001: 213 nM

PXL004: 37 nM

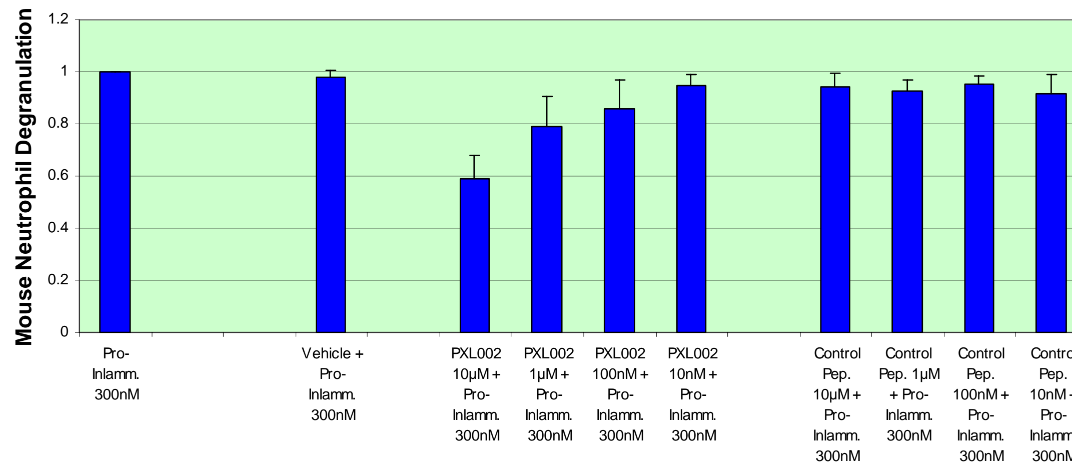
Inhibition of Human Monocyte Migration



PXL001: 2792 nM

PXL004: 710 nM

Inhibition of Mouse Neutrophil Activation



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Phage-Display / GPCRs – Reverse Pharmacology

- In-expensive method.
- Accessible to any laboratory (including Academic ones).
- Potent technique allowing the discovery of small specific peptides of high affinity for GPCRs (low nM range).
- After 3 pannings, more than 80 % of positive peptides.
- Independent of reference molecules.
- Tissue-specific libraries for 7TM de-orphanisation.
- Allow the discovery of ortho- and allo-steric molecules.
- Fast access to characterize the pharmacology of a dedicated target.
- Applicable to paired or orphan GPCRs.
- Useful in the context of hetero-dimers, -oligomers.

Development of New Luciferase Reporter Gene Assays for the Characterization of GPCR Constitutive Activity and/or for the Characterization of the Signaling of Orphan GPCRs

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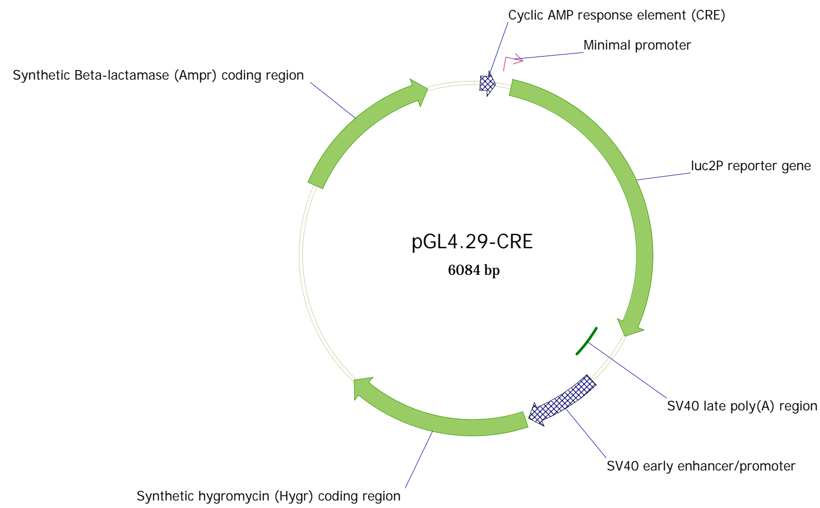
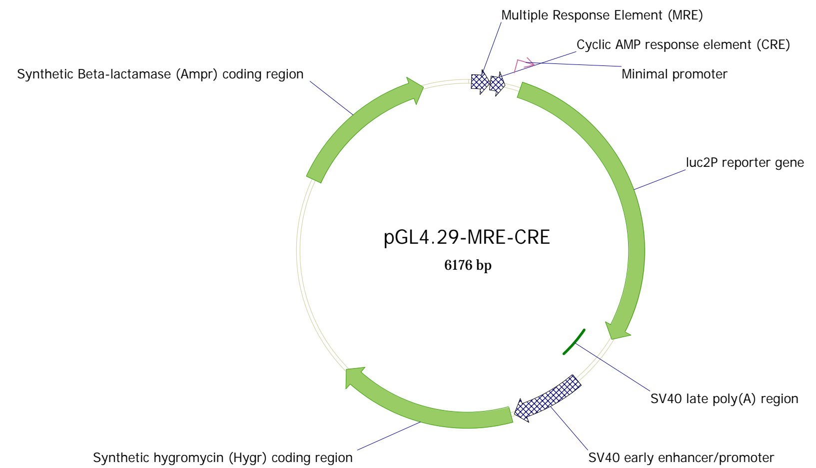
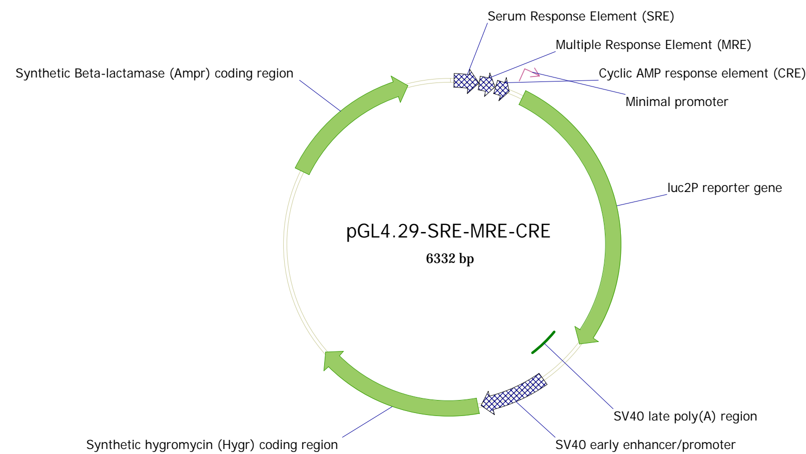
New Luciferase Reporter Gene Assays

- Search for a reporter system as larger as possible to find potential pathways after the activation of a 7TM.
- SRE, Serum Response Elements, are promoter elements required for the regulation of many cellular immediate-early genes by growth.
- MRE, Multiple Response Elements, are enhancer elements that confer responsiveness to multiple cytokines and second messengers.
- CRE, cAMP Response Element, is a transcription regulatory sequence that interacts with transcription factors which mediate signal transduction involving cAMP.
- Typically, in the universal GPCR reporter constructs, the response elements are operatively linked to a reporter gene that functions to identify constitutive activated 7TM, the presence or absence of ligand-activated 7TM. The reporter genes will be polynucleotides that encode selectable or a detectable polypeptides.
- Development of a system based on firefly luciferase, a minimal promoter (mIP), and 3xCRE, or 3MRE-3xCRE, or 3xSRE-3xMRE-3-CRE.

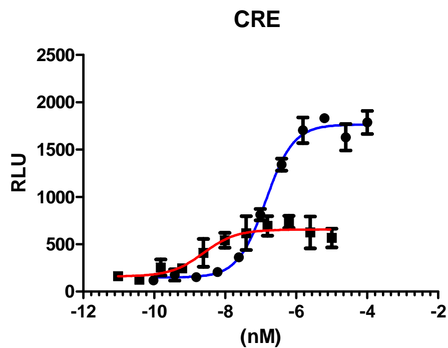
New Luciferase Reporter Gene Assays



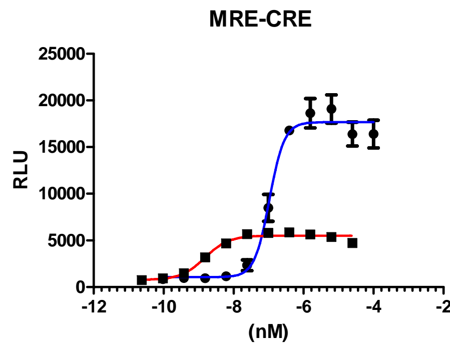




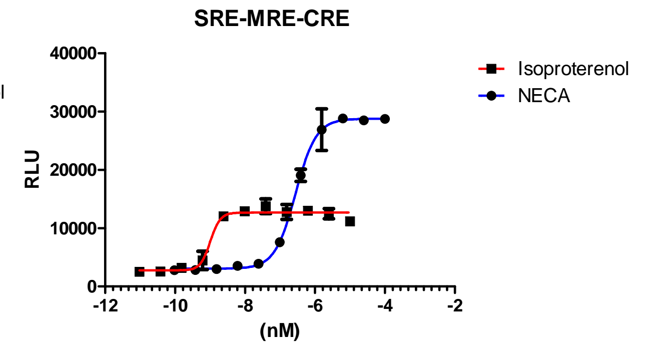
Response after Activation of Endogenous 7TMs



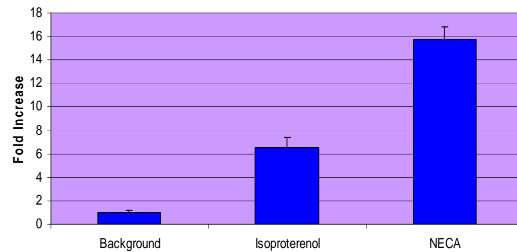
	NECA	Isoproterenol
EC50	1.381e-007	2.447e-009

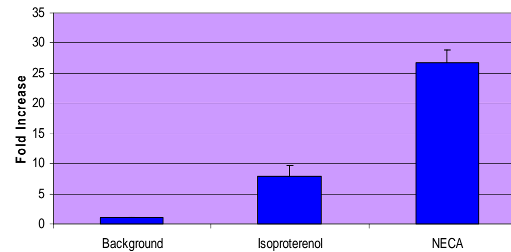


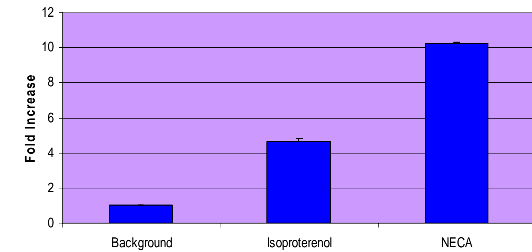
	NECA	Isoproterenol
EC50	1.064e-007	1.519e-009



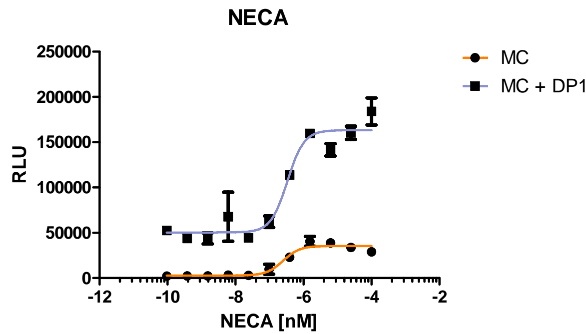
	NECA	Isoproterenol
EC50	2.773e-007	1.018e-009



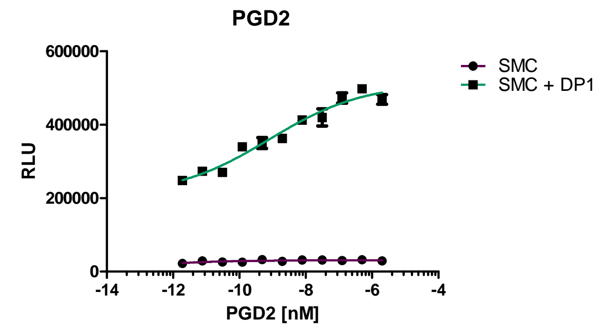




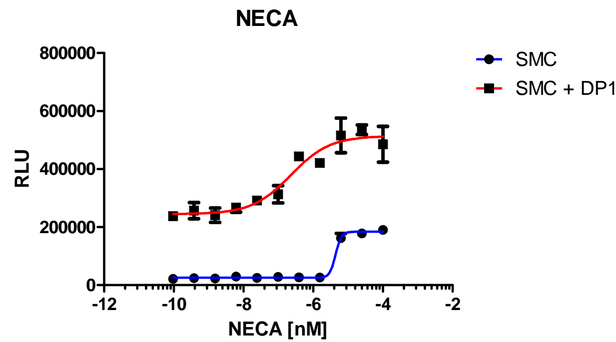
Response after Activation of Endogenous or Recombinant 7TMs



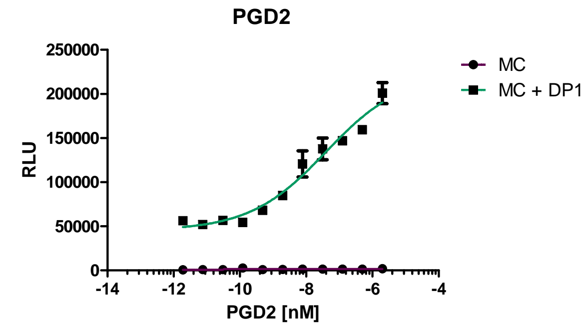
	MC	MC + DP1
EC50	2.521e-007	3.364e-007



	SMC	SMC + DP1
EC50	~ 0.0	7.967e-010



	SMC	SMC + DP1
EC50	4.383e-006	2.297e-007



MC	MC + DP1
~ 3.239e-011	3.951e-008

Multiplexing Calcium and Beta-arrestin Read-outs on FLIPR Tetra – Application to Screening and Profiling

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Multiplexing Calcium and Beta-arrestin Read-outs on FLIPR Tetra – Application to Screening and Profiling

- PathHunter HEK-GPCRX β-arrestin 2 (DiscoveRx) + transient Gα16
  - 1. Plate PathHunter HEK-GPCRX β-arrestin 2 transfected 48 h before with Gα16 (1 or 2 ug / well of a 6 well-plate) cells the day before the assay on 384 Greiner plates in 40 uL of complete medium without selection pressure.
  - 2. Aspirate to remove the medium.
  - 3. Add 30 uL of assay medium in each well (Fluo4-AM).
  - 4. Incubate 1h at 37°C / 5% CO2.
  - 5. Proceed to FLIPR measurement by adding 5 µl of a 7 fold concentrated compound solution per well.
  - 6. Incubate 1h at 37°C / 5% CO2.
  - 7. Proceed to PathHunter FLASH Detection Kit (Discoverx) measurement with FLIPR Tetra by adding 20 µl of detection reagent by well.

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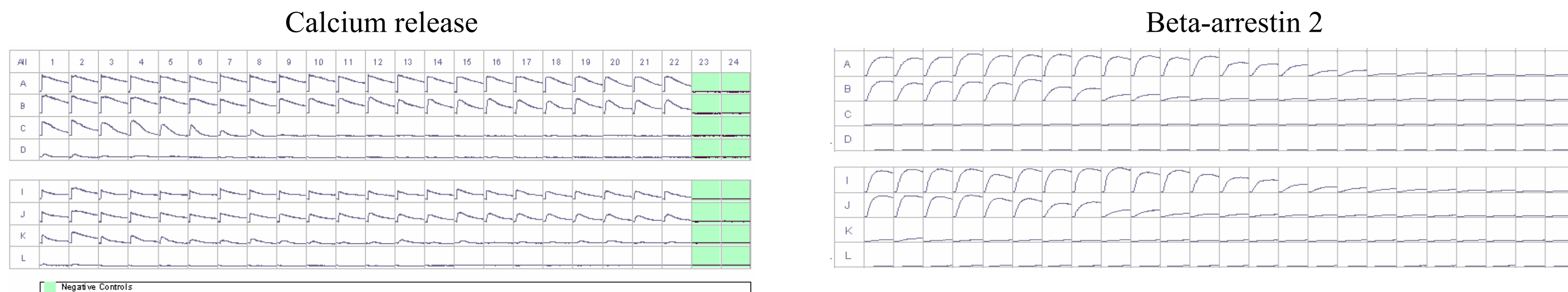
Multiplexing Calcium and Beta-arrestin Read-outs on FLIPR Tetra – Application to Screening and Profiling



- Sequential analysis on FLIPR tetra of Calcium release and beta-Arrestin recruitment in the same well .
- We were able to demonstrate that the same plate treated with compounds can be sequentially analyzed first for calcium mobilization and then for arrestin signaling using modified PathHunter cell lines.
- The left panel shows the calcium measurement in HEK-GPCRX-Ga16 stimulated with 3 ligands, and the right panel shows the arrestin response in the same cells (GPCRX is a Gi Coupled Receptor).
- Shift of activities were observed from Calcium to Beta-arrestin measurements.
- Compatible with quenchers of fluorescence.

Application of the FRET (SNAP-CLIP) Technology for the Characterization of GPCR Homo- and Hetero-dimers

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Application of the FRET (SNAP-CLIP) Technology for the Characterization of GPCR Homo- and Hetero-dimers

- Examples of G-protein-coupled receptors that can biochemically detected in homo- or heteromeric complexes are emerging at an accelerated rate.
- Biophysical approaches have confirmed the existence of several such complexes in living cells and there is a strong evidence to support the idea that dimerization is important in different aspects of receptor biogenesis and function.
- The existence of G-protein-couples receptor dimers raises fundamental questions about the molecular mechanisms involved in transmitter recognition and signal transduction.
- The formation of heterodimers raises fascinating combinatorial possibilities that could underlie an unexpected level of pharmacological diversity, and contribute to cross-talk regulation between transmission systems.
- Because GPCRs are major pharmacological targets, the existence of dimers could have important implications for the development and screening of new drugs.

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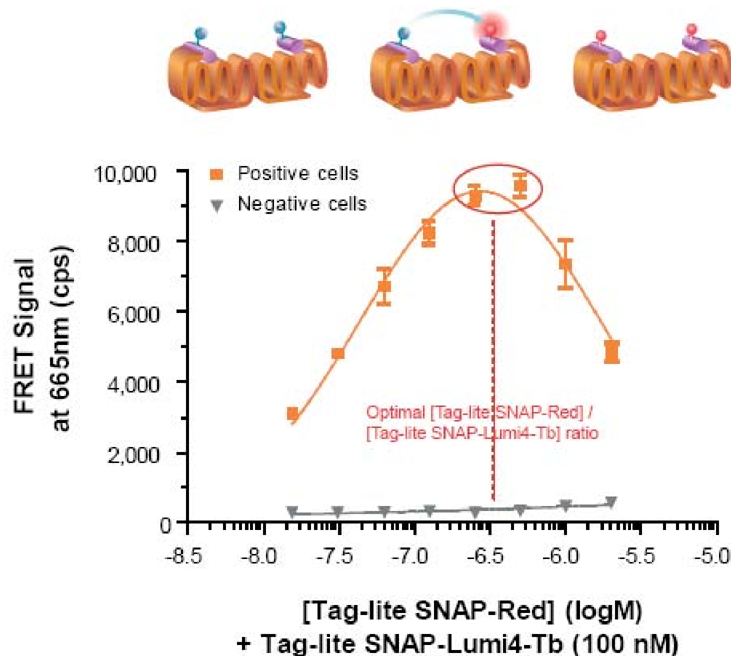
Application of the FRET (SNAP-CLIP) Technology for the Characterization of GPCR Homo- and Hetero-dimers

- Taste receptors
- $GABA_{B1}$-$GABA_{B2}$ receptors (G. Milligan, Br. J. Pharmacol., 158: 5-14, 2009)
- SSTR5-D2 receptor (Rocheville et al., Science, 288: 154-157, 2000)
- AT1R-B2 receptor (AbdAlla et al., Nat. Med., 7: 1003-1009, 2001)
- CB1R-OX1R (Hilairet et al., J. Biol. Chem., 278: 23731-23737, 2003)
- TRHR-OX1R (WO/2008/055314)
- GPR50-MT1R (Levoye et al., EMBO J., 25: 3012-3023, 2006)
- GPR17-CLT1R (Maekawa et al., Proc Natl Acad Sci U S A., 106:11685-11690, 2009)
- CXCR4-CXCR7
- Serotonin-Glutamate receptors (WO/2009/100384)

- And more than 30 others

Application of the FRET (SNAP-CLIP) Technology for the Characterization of GPCR Homo- and Hetero-dimers



- Fluorescence intensity at 665 nm (specific of the biological interaction) is plotted versus Taglite SNAP-Red concentration for both the positive and the negative conditions.
- The conditions to ensure equivalent labeling of the SNAP-tags with each fluorophore are defined after double-labeling of cells with both Tag-lite SNAP Lumi4-Tb and Tag-lite SNAPRed.
- The optimal [Tag-lite SNAP-Red]/[Tag-lite SNAP Lumi4-Tb] ratio is the one that gives the maximum FRET signal (hence the maximum 665 nm value).
- The specific 665 nm emission signal is directly proportional to the amount of dimers at the cell surface.

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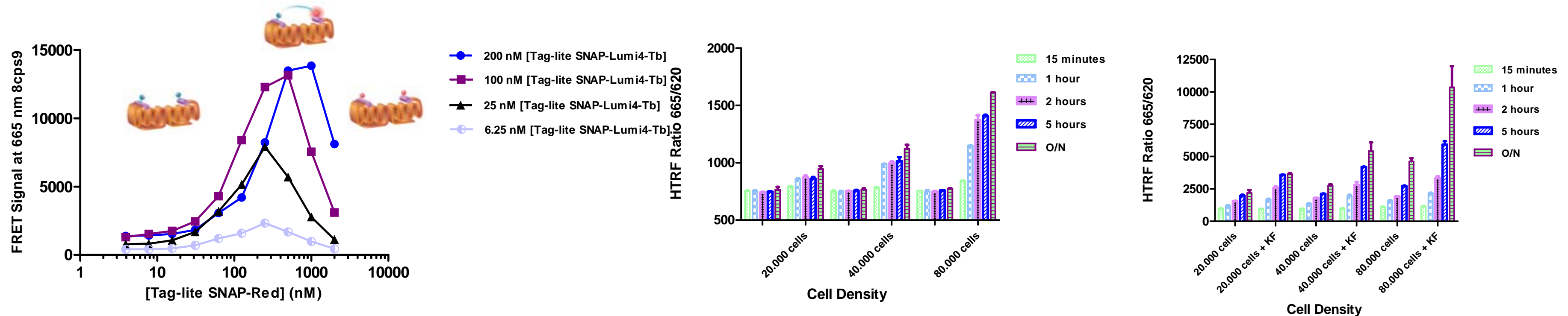
Application of the FRET (SNAP-CLIP) Technology for the Characterization of GPCR Homo- and Hetero-dimers



- Homo-dimerisation demonstrated by TR-FRET on recombinant cell-lines using:
  - SNAP-tag technology.
  - Monoclonal antibodies directed against HA-tags.
  - Monoclonal Antibodies directed against the N-terminal part of the 7TM.
- The availability of monoclonal Abs allows the translation to studies on human primary cells.

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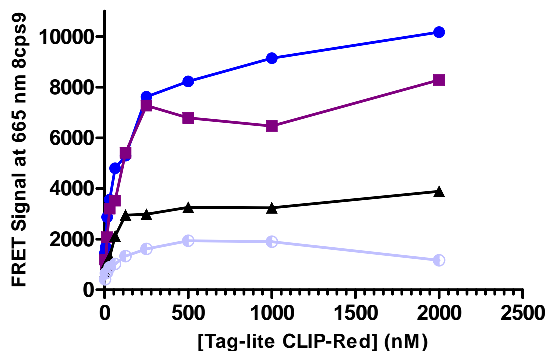
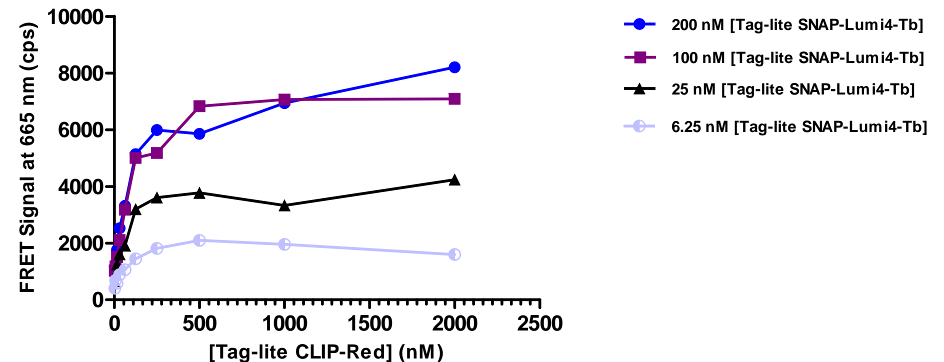
Application of the FRET (SNAP-CLIP) Technology for the Characterization of GPCR Homo- and Hetero-dimers

- We demonstrated that GPCRX receptors exist as homodimers, homo-oligomers.
- We demonstrated that GPCRY and GPCRZ also exist as homodimers, homo-oligomers.
- GPCRX and GPCRY:
  - twin receptors.
  - co-expressed in the same tissues, and in the same cells.
  - co-up-regulated in inflammatory conditions.

- Interest to search for hetero-dimers GPCRX-GPCRY:
  - Modulation of GPCRX might modulate GPCRY.
  - Internalization of GPCRX might de-activate GPCRY.
  - Better understanding of GPCRX pharmacology.
  - Motivation to search for other 7TM partners.

Application of the FRET (SNAP-CLIP) Technology for the Characterization of GPCR Homo- and Hetero-dimers





• GPCRY behaves as a “ligand” for GPCRX.

• Consequences of receptor oligomerization is a possible cross-talk between the protomers.

• We are performing experiments to demonstrate if negative and positive cooperative binding can be observed with different ligands on GPCRX and GPCRY receptors.

• Tools amendable for the replacement a radio-ligand displacement (Dr. Durroux’s presentation).

Application of the DiscovRx Beta-arrestin Cell-based GPCR Assay for Receptor De-orphanisation

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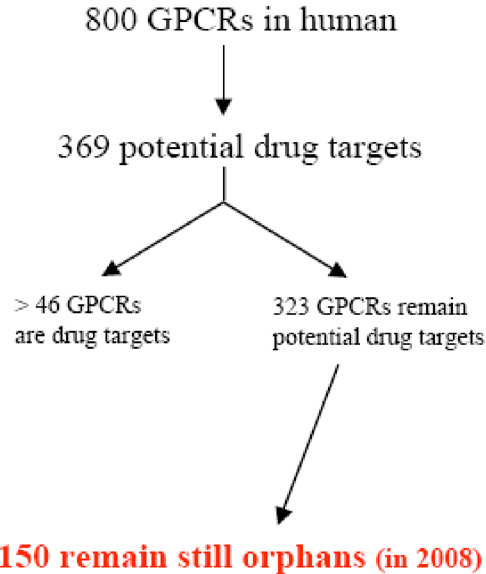
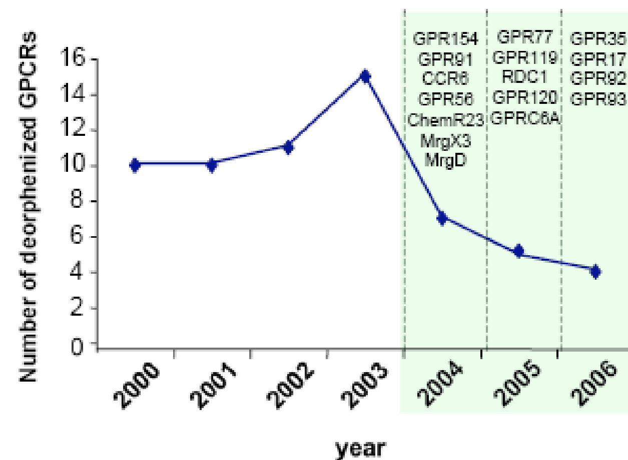
Application of the DiscovRx Beta-arrestin Cell-based GPCR Assay for Receptor De-orphanisation



Lagerström and Schiöth Nat Rev Drug Discov, 2008



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Application of the DiscovRx Beta-arrestin Cell-based GPCR Assay for Receptor De-orphanisation

- Use phylogenetic trees to find potential orphan targets, closed to our currently investigated 7TMs and/or to our known natural ligands to develop and create a leadership around our currently explored GPCRs.
- As GPCRX, GPCRY, and GPCRZ form homo-dimers and that we demonstrated that GPCRX-GPCRY hetrodimers exist, the second idea was:
  - Use the phylogenetic tree to find potential hot partners for GPCRX,Y and Z receptors (hetreo-dimers), including potential ligands of GPCR heterodimers.
- As most of the deorphanised GPCRs are patented, it is important to be the first on new important targets.
- Division arrested cell-lines acquired from DiscoverX.
- Passage limited: once in culture, use in the 2 days.
- Evaluation of potential ligands on beta-arrestin 2 signaling based on PCA technology.

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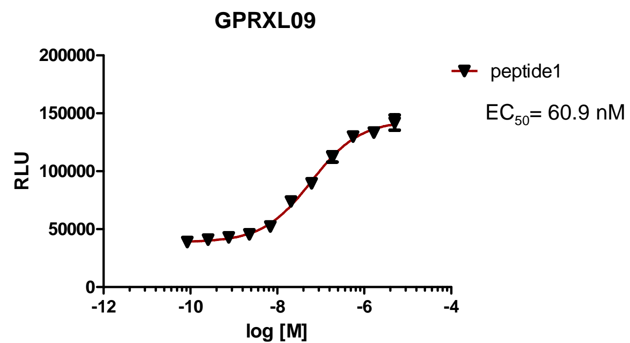
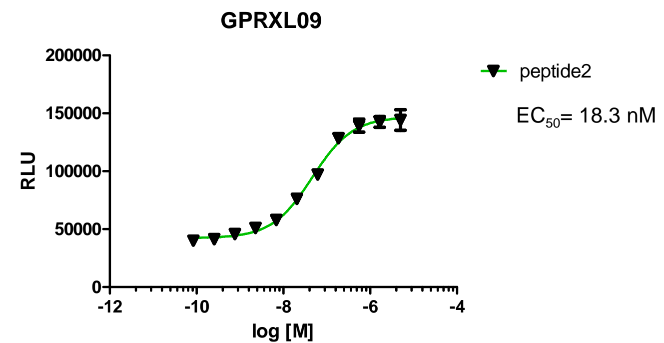
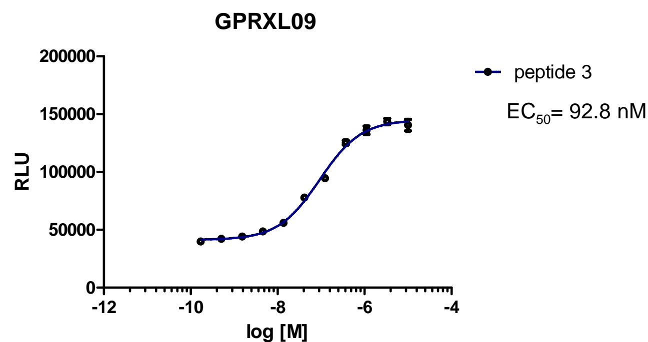
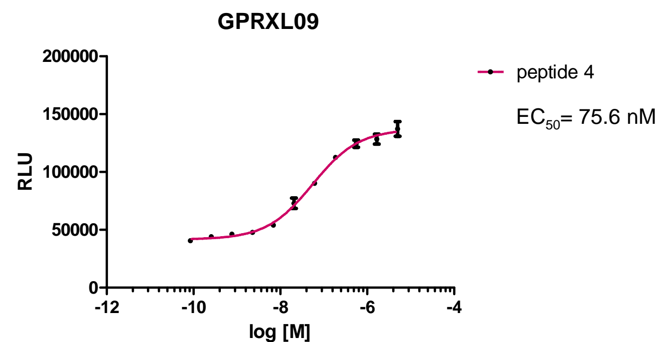
Application of the DiscovRx Beta-arrestin Cell-based GPCR Assay for Receptor De-orphanisation









- Fast approach for GPCR de-orphanisation
- Arrestin “specific” pathway(s)

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Conclusions

- SNAP- & CLIP-tags suicide enzymes can easily be fused to either the N- or the C-terminal position on 7TMs and can be specifically and covalently labeled with their specific substrates. This platform is an original way to study:
  - 7TM expression,
  - 7TM internalization,
  - Binding avoiding radiolabeled molecules,
  - GPCR-GPCR interactions.
- PCA, as beta-galactosidase applied to monitor the recruitment of beta-arrestin by 7TM, appears as a method of choice to study highly important signaling pathways, the pairing of orphan GPCRs, and the study of receptor dimers.
- Multiplexing on FLIPR tetra: possible for many applications, especially regarding G protein and arrestin pathways.
- Be inventive and not waiting for tools that are provided to everybody. Making the differences by applying to 7TM tools developed for other targets and other applications:
  - Phage-display on GPCR expressed on rec. systems,
  - Multiple responding elements,
  - Other PCAs.

Thank you on behalf of all Actelion employees