Direct Fluorescent labelling approaches for G-protein coupled receptors using the ImageXpress$^{ULTRA\ TM}$

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1 Introduction

One of the most powerful tools for receptor research and drug discovery is the study of receptor-ligand interactions.

Historically, radioactive ligands have been used to identify a binding event; however, there are numerous limitations involved in the use of radioactivity for high-throughput screening.

The recent development of high-throughput confocal imaging plate readers such as the ImageXpress^{ULTRA} ™ from MDS Analytical Technologies and the concurrent development of high affinity fluorescent ligands which selectively bind to G-protein coupled receptors (GPCRs) has provided a novel and non-radioactive alternative for the determination of ligand binding affinities.

In this study we have developed a ligand binding assay which takes advantage of both high affinity fluorescent ligands and imaging technology to determine the ligand –binding characteristics at five functionally and structurally distinct GPCRs (the adenosine A1, the muscarinic M3, the dopamine D1, the histamine H2 receptor and the β2-adrenoceptor (β2-AR)).

Collectively these data highlight a novel and quantitative approach to the determination of ligand binding affinities.

2 Methods

Cells were grown to confluence in 96-well black view plates (Corning Co-star) in DMEM/F12 medium supplemented with 10% fetal calf serum and 2mM glutamine at 37°C, 5% CO_2/humidified air.

- On the day of experimentation all media were removed and the cells stained with 1μg/ml Hoechst stain in hepes buffered saline (HBS) for 10 minutes at room temperature.
- Cells were washed once with HBS and resuspended in a final volume of 100μl/well HBS.
- Unlabelled competitors were pre-incubated on cells for 30 minutes at room temperature prior to the addition of a receptor selective fluorescent ligand (Table 1; CellAura Technologies Ltd.).
- Fluorescent ligand binding was detected over a 10 minute period on the ImageXpress ^{Ultra} ™.
- Fluorescent ligands were excited by a 633nm laser. The Hoechst stain was excited by a 405nm laser.

3 Imaging data

- Fully automated point-scanning confocal imaging system.
- Up to 4-solid state lasers for simultaneous or sequential scanning.
- User selectable self-aligning main beam-splitters.
- Dedicated high-speed laser auto-focus to minimize sample bleaching.
- 4-position automated objective changer.
- Up to 3 user-selectable filter sets.
- Z focus with better than 100nm resolution.
- Software-configurable detection pinhole diameter.

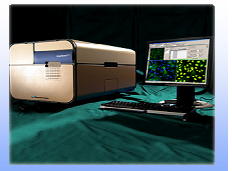

Figure 1: ImageXpress ^{Ultra} ™

4 Results

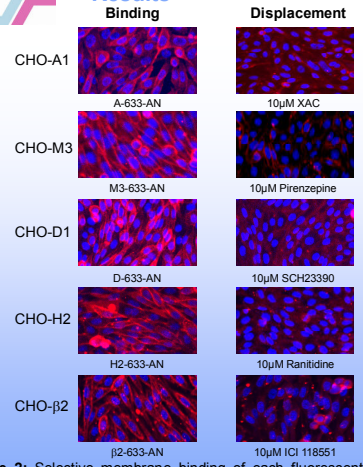

Figure 2: Selective membrane binding of each fluorescent ligand was detected at a final concentration of 100nM in CHO cells stably expressing the target receptor. Selective membrane binding of the fluorescent ligand was displaced by a 30 minute pre-incubation with an unlabelled competitor (final concentration of 10μM).

5 Image Analysis

- Membrane bound fluorescence was quantified using the MetaMorph ™ analysis software from MDS Analytical Technologies.
- The signal to noise ratio was improved by filtering each image through a ranking algorithm. A rank was applied to each pixel with the background representing the lowest rank.
- A threshold was then applied to each image to eliminate background fluorescence while identifying membrane bound fluorescence.
- The integrated intensity was then calculated within a given area of each image.

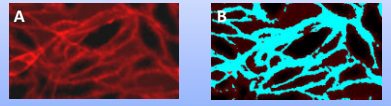

Figure 3: MetaMorph™ analysis of membrane bound β2-633-AG fluorescence in CHO cells stably expressing the β2-AR. By applying a threshold to the ranked imaged (A) only membrane bound fluorescence is identified hence eliminating any background or non-membrane bound fluorescence (B).

6 Saturation Binding Data

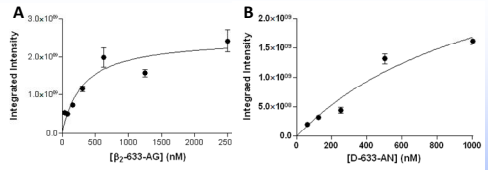

Figure 4: Specific binding of β_2-633-AG (A) and D-633-AN (B) was determined in CHO cells stably expressing the human β_2-AR or the dopamine D1 receptor respectively. Specific binding was taken as that displaceable by 10μM unlabelled ICI 118551 (β_2-AR) or 10μM SCH 23390 (dopamine D1 receptor). Whole cell saturation binding studies on CHO-β2 cells gave a $\log K_D$ value of -6.49 (β_2-633-AG) while studies on CHO-D1 cells gave a $\log K_D$ value of -5.92 (D-633-AN).

7 Competitive Binding

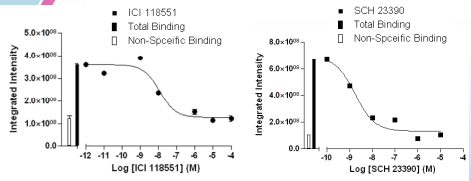

Figure 5: The β2-selective antagonist ICI 118551 decreased binding of 100nM β2-633-AG to the β_2-AR in a dose dependent manner (A). Unlabelled ICI 118551 decreased β2-633-AG binding to the β_2-AR by 98.21 ± 5.61%. A $\log K_i$ value for this event was calculated at -9.07 ± 0.45. The selective D1-antagonist SCH 23390 decreased binding of 100nM D-633-AN to the D1-receptor in a dose dependent manner (B). Unlabelled SCH 23390 decreased D-633-AN binding by 105.66 ± 3.51% and producing a $\log K_i$ value of -8.41 ± 0.32.

8 Ligand Portfolio

Receptor	CellAura Ligand	Antagonist
Adenosine A1	A-633-AN	XAC
Dopamine D1	D-633-AN	SCH 23390
Histamine H2	H2-633-AN	Ranitidine
Muscarinic M3	M3-633-AN	Pirenzepine
β_2-Adrenoceptor	β2-633-AN	ICI 118551
β_2-Adrenoceptor	β2-633-AG	ICI 118551

Table 1: Description of all receptors reviewed in this study including their corresponding fluorescent ligands and unlabelled competitors. CellAura Technologies offers a wide range of high affinity fluorescent ligands for GPCRs which have comparable pharmacological properties to the parent ligand.

9 Discussion

These data indicate that ligand binding assays based on the compatibility between fluorescent ligand technology and the ImageXpress ^{Ultra} ™ provide a novel approach for the characterisation of the receptor-ligand interaction and highlight its future application in drug discovery.

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