Direct Fluorescent Labelling Approaches for G-Protein Coupled Receptors and Imaging using the Opera™ High Content Screening Platform

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1 Introduction

G-protein coupled receptors (GPCRs) continue to be an attractive drug target for a number of clinically relevant disorders. This is primarily due to their membrane location, ubiquitous expression and their involvement in virtually every mammalian physiological system. While a number of assays targeted at downstream GPCR signalling events have allowed characterisation of this receptor superfamily, few non-invasive assays allow analysis of the receptor-ligand interaction at the cellular level. Fluorescence microscopy provides a non-destructive and sensitive technique to visualise protein movements and cell function. In more recent years the development of high throughput confocal imagers which combine high-resolution fluorescence microscopy with automated image analysis has provided a sensitive platform on which to monitor ligand induced cell changes in a quantitative manner. The Opera™ System from PerkinElmer provides a sensitive screening platform enabling simultaneous image acquisition and quantification of cell-based assays at the cellular and sub-cellular levels. In this study we describe high affinity fluorescent ligands (CellAura Technologies Ltd.) which selectively target GPCRs and when used in combination with the Opera™ System provide a novel tool for the determination of ligand binding characteristics at a given receptor.

2 Methods

Experiments were performed on CHO cells stably expressing the human adenosine A1, dopamine D1, histamine H2 or muscarinic M3 receptors (PerkinElmer Cellular Sciences, Belgium, SPRL) or HEK cells stably expressing the human β_2-adrenoceptor (PerkinElmer CT, Germany). Cells were grown to semi-confluence in 384-CellCarrier™ plates in DMEM/F12 medium supplemented with 10% fetal calf serum at 37°C, 5% CO_2/humidified air. All experiments were performed in HEPES buffered saline (HBS) at room temperature. Cells were stained with the nuclear stain, Hoechst, for 10 minutes at room temperature at a final concentration of 1μg/ml in HBS. Cells were washed once with HBS and resuspended in a final volume of 30μl/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.). Following the addition of fluorescent ligand, membrane binding was detected over a 20 minute period on the Opera™. The Hoechst nuclear stain was excited by the 405nm laser, fluorescence was collected through 450/50 bandpass filter_. The fluorescent ligands were excited by the 635nm laser, fluorescence was collected through 690/50 bandpass filter.

3 Opera™ HCS Platform

- Fully automated, true multipoint confocal Nipkow disc scanning imaging reader
- 4 laser based excitation sources (405, 488, 561, 635 nm)
- 3 independent CCD detectors
- Fast laser-based autofocus
- Water immersion and air objectives
- Non-confocal UV option with 4th CCD camera
- Climate control option
- Dispensing option for kinetic applications
- Compatible with all plate formats
- High speed data acquisition and processing (up to 100,000 images in 24 h)

Figure 1: Opera™

4 Results

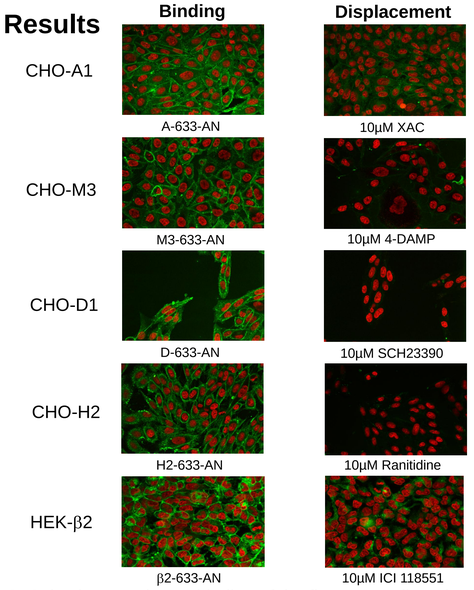

Figure 2: Selective membrane binding of the fluorescent ligands was detected at a final concentration of 30nM in CHO/HEK cells stably expressing the specific receptor. Selective membrane binding of the fluorescent ligand was displaced by a 30 minute pre-incubation with an unlabelled competitor (final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

5 Acapella™ Image Analysis

The Acapella™ image analysis platform allows precise quantification of the location, shape, structure and amount of fluorescence on an individual cell level. A set of readymade analysis algorithms - known as “Scripts” - are available for a broad range of standard situations like counting, translocation, spot analysis, etc. These Scripts have been used to create the data presented here. Due to its high speed Acapella™ is ideally suited for on the fly analysis and analysis of large data sets. Especially the Columbus™ data management system fully utilizes the power of Acapella™.

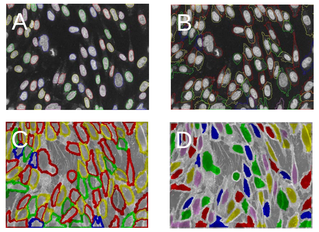

Figure 3: Acapella™ analysis of membrane bound and cytoplasmic fluorescence. The nucleus (A) and cytoplasm (B) are identified by defined algorithms. Once the cell membrane (C) and cytoplasm (D) have been identified, the degree of fluorescence in these areas can be determined.

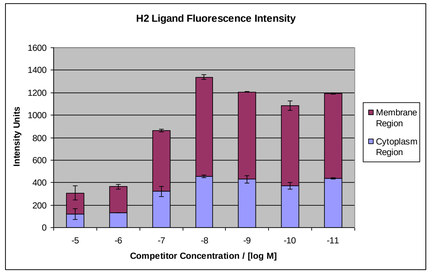

Figure 4: The Histamine H2-selective antagonist Ranitidine decreased the binding of H2-633-AN to the histamine H2-receptor in a dose dependent manner.

6 Kinetics of the A1-Ligand Binding:

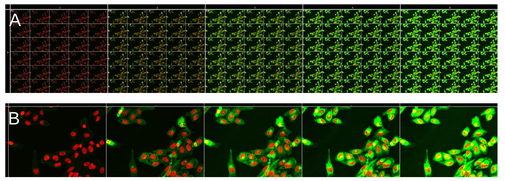

Figure 5: Combination of two kinetic measurements. Fluorescent A-633-AN ligand was added to CHO A1 cells and an automated kinetic measurement series of 30 images/min was repeated every five minutes. Each block of panel (A) shows a montage of thumbnail representations of the 30 images, indicating no significant changes within one minute, but strong increase of the overall fluorescence on the 20 minutes timescale. Panel (B) shows an enlarged view of one image of each block. The Acapella™ analysis reveals a linear increase of the fluorescence in time, no saturation is seen within 20 minutes. Images were captured with Olympus 40xPLANAPO water lens.

7 CellAura Technologies 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	4-DAMP
β_2-Adrenoceptor	β2-633-AN	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.

8 Discussion

These data indicate that ligand-binding assays based on the compatibility between fluorescent ligand technology and the Opera™ High Content Screening Platform provide a novel approach for the characterisation of the receptor-ligand interaction and highlight its future application in drug discovery.

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