CypHer™5 - A Generic Technology for the Measurement of Cell Surface Receptor Activation in Live Cell Assays

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INTRODUCTION

Cell surface receptors, consisting of sensitive cyanine dye derivative, which is designed to fluoresce only in an acidic environment. It is therefore ideally suited for monitoring agonist-induced entry of cell surface receptors into the endosomal pathway. Receptor activation of G-protein-coupled receptors (GPCRs) and the receptor tyrosine kinase (RTK), epidermal growth factor receptor (EGFR), were monitored in live cell assays using lamp based and laser based high throughput automated fluorescence microscope platforms (IN Cell Analyzer 1000 and IN Cell Analyzer 3000 respectively). The GPCRs were selected as examples of the sub-families that function predominantly via the elevation of intracellular cAMP (β2 adrenergic receptor), inhibition of cAMP production (α2 adrenergic receptor) or elevation of intracellular Ca²⁺ levels (TRH R-1).

1) Receptor Internalisation Assay

CypHer 5 is a red-excited fluorogenic cyanine dye that is non-fluorescent at pH 7.4, but fluorescent under acidic conditions when fully protonated which is ideal for measuring a translocation of the receptors.

Cell surface receptors (GPCRs/RTKs) are major drug targets used by the pharmaceutical industry. Agonist activation of these receptors almost inevitably results in the internalisation of the receptor, from the plasma membrane to the endosomal pathway within the cell. By N-terminally tagging a cell surface receptor with an epitope (or using an endogenous epitope), and labeling an antibody to that epitope with CypHer 5, the internalisation of a cell surface receptor can be monitored within the cell. CypHer 5 co-localisation experiments with a C-terminally GFP-tagged β2 adrenergic receptor after isoproterenol treatment, with a marked increase in CypHer 5 fluorescence (Figure 1).

2) In Cell Analyzer 1000 and In Cell Analyzer 3000:- High Throughput automated cellular imaging systems.

Cellular assays are fast becoming a major requirement for screening and drug profiling within the drug discovery pipelines. Amersham Biosciences have two instrument platforms (IN Cell Analyzer 1000 and IN Cell Analyzer 3000) that enable high throughputs automated cellular imaging. The IN Cell Analyzer 1000 is a fast lamp based epi-illumination microscope system and provides flexible assay development for cellular analysis. The IN Cell Analyzer 3000 is a fast laser based confocal system which results in increased sensitivity and enables assay throughputs of up to 30,000 wells per day.

This study shows that CypHer 5 can measure cell surface receptor activation on both the IN Cell Analyzer 1000 and IN Cell Analyzer 3000 and therefore provides a generic technology that can be applied to both lamp based and laser based imaging systems.

3) Monitoring GPCR receptor internalisation using CypHer

CypHer 5 provides a method with which to monitor the activation of G-protein coupled receptors (and other internalised cell surface receptors). In order to validate this platform, CypHer 5 was applied to GPCRs spanning the following sub-classes, (β-2 adrenergic), (α2 adrenergic), (β2-adrenergic) (Opioid receptor) and the receptor tyrosine kinase EGFR. Stable cell lines were generated that expressed recombinant DNAs containing a N-terminal epitope tag (VSV-G) fused to the receptors above. Cells were pre-incubated with either 5µM CypHer 5 labelled anti VSV-G antibody (clone P8) or the anti-EGFR antibody (clone ICR10) and then stimulated with the appropriate agonist to activate the receptor. Cells were imaged for red CypHer 5 fluorescence using the IN Cell Analyzer 3000, 633nm laser line and the 670nm excitation wavelength for the IN Cell Analyzer 1000.

4) In Cell Analyzer 3000

4.1) Internalisation of the β2 adrenergic receptor (β2-AR)

Translocation to the perinuclear recycling endosomes was observed in HEK293 cells expressing a VSV-G tagged β2 adrenergic receptor after isoproterenol treatment, with a marked increase in CypHer 5 fluorescence (Figure 1).

4.2) Internalisation of the TRH receptor (Gq)

Chinese Hamster Ovary (CHO) cells expressing the VSV-G TRHR were treated with various concentrations of TRHR (0-10µM) for 30 minutes, following pre-incubulation with CypHer 5 labelled antibody. Translocation to the perinuclear recycling endosomes was observed, similar to figure 1 (data not shown). The data were analysed using the Granularity Analysis module and a sigmoidal dose response curve with an EC50 value of 10.4nM was observed (Figure 5).

5) IN Cell Analyzer 1000

5.1) Internalisation of the α2-adrenergic receptor (α2-AR)

Chinese Hamster Ovary (CHO) cells expressing the α2-ADREnergic receptor after incubation with 1µM Agenst (DADLE) for 40 minutes. The measurement of increasing signal intensity of CypHer 5-internalised cell surface receptors. The data were analysed using the Granulatrity Analysis algorithm and a sigmoidal dose response curve with an EC50 value of 1.4nM was observed (Figure 3).

5.2) Internalisation of the EGFR receptor

To demonstrate that CypHer 5 is not restricted to GPCRs and can measure the receptor activation of other cell surface receptors, the internalisation of the Epidermal Growth Factor Receptor, a receptor tyrosine kinase was monitored. HEK293 cells expressing the EGFR receptor were treated with various concentrations of EGF, following pre-incubulation with CypHer 5 conjugated to the anti-EGFR antibody ICR10 (Serotec). Translocation was observed, similar to figure 4 (data not shown). The data were analysed using the Granulatrity Analysis algorithm and a sigmoidal dose response curve with an EC50 value of 0.41nM was observed (Figure 6).

Conclusions:

- A generic assay with which to monitor the activation of GPCRs and RTKs in a physiologically relevant live cell based assay
- An assay that is applicable to both lamp based and laser based high throughput cellular imaging platforms
- A new approach for functional analysis and screening of GPCRs