MINIATURIZATION OF A HOMOGENEOUS FLUORESCENCE POLARIZATION ASSAY FOR M_1 MUSCARINIC ACETYLCHOLINE RECEPTORS USING CyDye LABELLED NON-PEPTIDE LIGANDS

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Introduction

Fluorescence polarization (FP) has successfully been applied to receptor ligand binding assays[1]. As a homogeneous assay system the potential of FP for assay miniaturization in order to increase throughput and reduce reagent costs for high throughput screening (HTS), without compromising assay reliability is worthy of investigation.

We have previously developed a 96-well homogeneous FP assay for the binding of telenzepine, a non-peptide antagonist, to Chinese Hamster Ovary (CHO) cells expressing M_1 muscarinic acetylcholine receptors (M_1AchR)[2]. Here we describe how the use of CyDye™ fluorescent labels and FARCyte™ fluorescence plate reader have enabled successful conversion of this FP assay to 384-well and 1536-well microtitre plate formats.

Method

Activated esters of Cy™5 and Cy3B were individually coupled to telenzepine amine congener‡. The affinities of the fluorescently labelled ligands (Table 1) were determined by radioactive competition binding assay and IC_{50} values compared with unlabelled telenzepine ligands as described previously[2].

Table 1. Telenzepine ligand IC_{50} values.

Compound	IC_{50}
Telenzepine diHCl	20nM
Telenzepine amine congener (TAC)	28nM
Cy5-telenzepine	120nM
Cy3B-telenzepine	13nM

$CHOM_1$ membranes (15µg) were incubated in the presence of 0.4nM L-Quinuclidinyl[*phenyl*-4-^{3}H]benzilate ([^{3}H]QNB) with varying concentrations of the following competitors: telenzepine, TAC, Cy5-telenzepine and Cy3B-telenzepine. Non-specific binding was determined in the presence of 100 µM atropine. Incubations were performed at room temperature for 3 hours in 50mM Tris, pH7.5 containing 0.1% (w/v) BSA and 5mM $MgCl_2$. Receptor bound ligand was separated from free by filtration using the Skatron Micro96 harvester. After sealing the filtermat onto Meltilex scintillant, counts were determined using an EG&G Wallac, 1205 Betaplate scintillation counter.

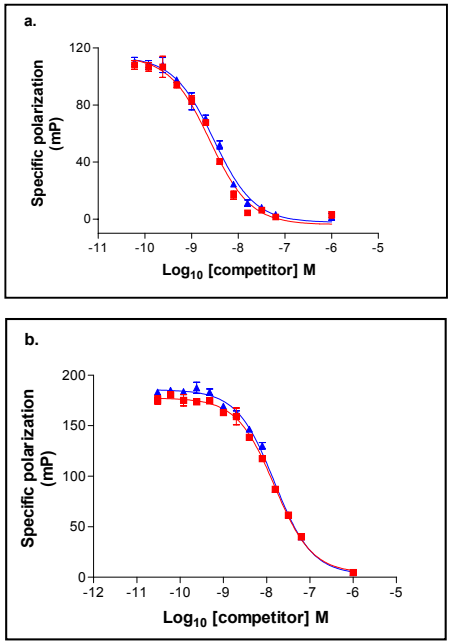
For 384-well FP assays, $CHOM_1$ cell membranes (from cells expressing the M_1 muscarinic receptor at ~10pmols/mg protein) were incubated with either 1nM Cy5-telenzepine or Cy3B-telenzepine in the presence of varying amounts of either unlabelled atropine or telenzepine for 4 hours at room temperature (20-25°C) in 50mM Tris buffer pH7.5 containing 0.1% (w/v) BSA and 5mM $MgCl_2$. Incubations were performed in a total volume of 50µl in Corning, non-binding surface, black 384-well plates. Non-specific binding (NSB) was determined in the presence of 100µM atropine. Fluorescence polarization was then determined on FARCyte using Cy5 or Cy3B optical filter/dichroic configurations as appropriate. Specific polarization values were calculated by subtracting the polarization signal obtained in the presence of 100µM atropine from the B_0 values.

For the 1536-well microtitre plate assays, 1µg $CHOM_1$ cell membranes were incubated with 0.5nM Cy3B-telenzepine, as described above, in a total assay volume of 8µl in Greiner 1536-well plates.

Results

Maximal change in the FP signal upon displacement was obtained using Cy3B-telenzepine in the 384-well assay but consistent results were observed with both Cy3B and Cy5 labelled ligands (Figure 1). Similar performance of the Cy3B assay was also observed during further miniaturization of this assay to a 1536-well format (Figure 3).

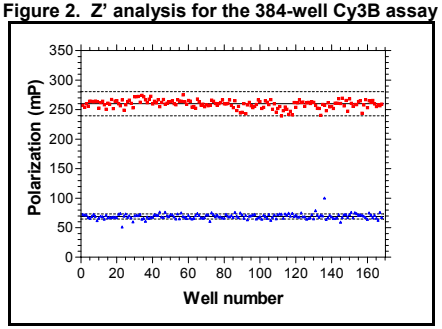
Figure 1. M_1AchR competitive binding assays (with atropine and telenzepine) in 384-well FP format



(a) $CHOM_1$ cell membranes (30µg) were incubated with 1nM Cy5-telenzepine or, (b) $CHOM_1$ cell membranes (15µg) were incubated with 1nM Cy3B-telenzepine, and either atropine (■) or unlabelled telenzepine (▲) as described in the text and FP data was subsequently measured on FARCyte. Values are plotted as means of quadruplicates ± SEM.

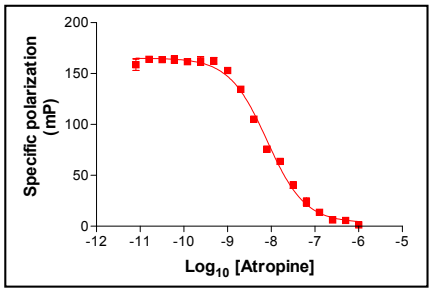
Z' factor analysis, recently described by Zhang *et al* [3], describes assays with a Z' factor between 0.5 and 1.0 to be reliable and robust for HTS. In Cy3B assays we have established values of 0.82 and 0.62 for the 384 and 1536-well formats respectively (Figures 2 and 4), suggesting both to be suitable for HTS purposes.

Figure 2. Z' analysis for the 384-well Cy3B assay



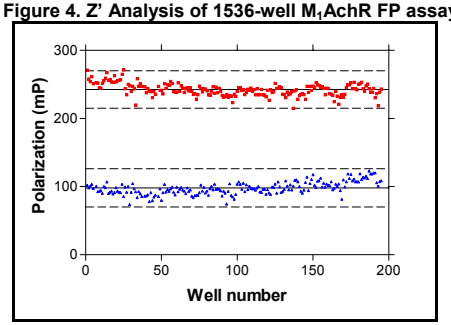
$CHOM_1$ cell membranes (15µg) were incubated with 1nM Cy3B-telenzepine in the absence (■) or the presence (▲) of 100 µM atropine as described in the text and FP measured on 168 replicates of each on FARCyte.

Figure 3. M_1AchR competitive binding assays (with atropine) in 1536-well FP format



$CHOM_1$ cell membranes (1µg) were incubated with 0.5nM Cy3B-telenzepine and varying concentrations of atropine as described in the text. FP was measured on the FARCyte instrument. Values are plotted as means of quadruplicates ± SEM.

Figure 4. Z' Analysis of 1536-well M_1AchR FP assay



$CHOM_1$ cell membranes (1µg) were incubated with 0.5nM Cy3B-telenzepine in the absence (■) or the presence (▲) of 100 µM atropine as described in the text and FP measured on 192 replicates of each on FARCyte. We have developed this 1536-well plate assay by hand and there are many important "sample handling" factors that would require further investigation for configuration of the assay in a full screening format.

Table 2 compares the different assay formats employed for the telenzepine, M_1AchR assay, using both Cy3B and Cy5 labelled ligands. Miniaturization represented an overall 40 – 50 fold reduction in ligand/membrane usage and 25 fold reduction in assay volume, whilst retaining the ability to monitor competitive displacement and maintaining acceptable assay reliability.

Table 2: Summary of FP assay miniaturisation.

(a) Cy3B assay conditions	96-well	384-well	1536-well
Membrane protein	50µg	15µg	1µg
Cy3B-telenzepine	160fmol	50fmol	4fmol
Volume of the assay	200µl	50µl	8µl
Conc. of Cy3B telenzepine	0.8nM	1.0nM	0.5nM
Apparent IC_{50} (atropine)	4nM	15nM	8nM
Assay Z' factor	nd	0.82	0.64

(b) Cy5 assay conditions	96-well	384-well
Membrane protein	50µg	30µg
Cy5-telenzepine	600fmol	50fmol
Volume of the assay	200µl	50µl
Conc. of Cy5 telenzepine	3.0nM	1.0nM
Apparent IC_{50} (atropine)	2.5nM	2.6nM

Values quoted for (a) Cy3B-telenzepine and (b) Cy5-telenzepine assays performed in 96-well plates are determined from data previously published in reference 2.
nd = not determined. Apparent IC_{50} values are determined as 50% displacement values from the specific polarization competition curves described in the text and not from further data analysis or derivation.

CONCLUSIONS

- We have successfully miniaturized an FP receptor ligand assay to 384 and 1536-well formats using CyDye labels and FARCyte.
- The assay miniaturization significantly reduced reagent consumption without compromising assay reliability.
- Consistent apparent IC_{50} values were obtained in all plate densities and Z' values of >0.5 were also observed.
- We have used the telenzepine, M_1AchR assay as a model system, but the same approach could be applied to other small molecule receptor assays, using CyDye labeling and detection on FARCyte fluorescent plate reader.

References:

1. Tota, M.R., *et al* (1995), *J. Biol. Chem.*, **270** (44), 26466 – 26472.
2. Harris, A.J., *et al* (1999), Poster presentation, annual meeting of the *Soc. of Neuroscience.*
3. Zhang, J-H., *et al* (1999), *J. Biomol. Screening*, **2**, 67 - 73.

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