A HOMOGENEOUS FLUORESCENCE POLARIZATION ASSAY FOR NEUROKININ-1 RECEPTORS USING Cy3B LABELLED SUBSTANCE P

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Introduction

Fluorescence Polarization (FP) is a homogeneous technique that is now widely used for high throughput screening (HTS). We have labelled Substance P (Sub P) with Cy™3B at the N-terminus or at the internal lysine position (see below). These peptide ligands together with FARCyte™ fluorescent plate reader have been used to develop 384-well and miniaturized 1536-well FP assays for the Neurokinin-1 (NK_1) receptor.

Cy3B-(terminal)-Sub P: Cy3B-RPKPQQFFGLM
Cy3B-(lysine)-Sub P: RPK(Cy3B)-PQQFFGLM

Method

Peptides were prepared by automated synthesis where acetylation at the N-terminus or ε-lysine facilitated site specific labelling. Cy3B was introduced via *in situ* activation and the labelled peptides purified by conventional reverse phase HPLC. The lyophilized products were characterized by UV spectroscopy and Maldi Tof mass spectroscopy. The affinities of Cy3B labelled Sub P ligands for the NK_1 receptor (Table 1) were determined by radioactive Scintillation Proximity Assay (SPA) and compared with the native ligand.

Table 1: Substance P ligand IC_{50} values.

Compound	IC_{50}
Sub P	0.17nM
Cy3B-(lysine)-Sub P	5nM
Cy3B-(terminal)-Sub P	2nM

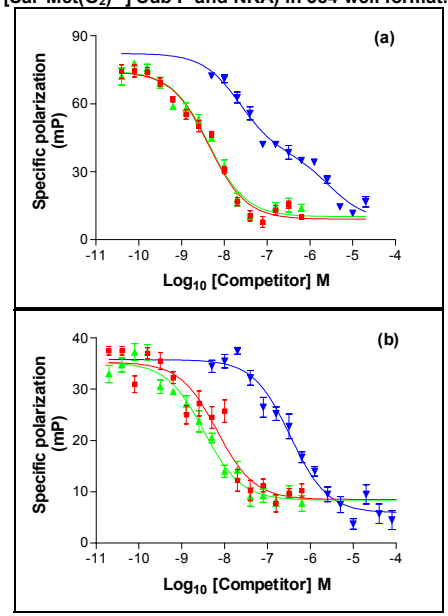
CHO NK_1 membranes (5µg) were incubated in the presence of 70pM [^{125}I]Sub P with varying concentrations of competitors; Sub P, Cy3B-(lysine)-Sub P or Cy3B-(terminal)-Sub P together with 0.5mg Type B PEI WGA SPA beads. Non-specific binding was determined in the presence of 25µM Sub P. Incubations were performed at room temperature for 3 hours in 50mM Tris, pH7.5 containing 0.5%(w/v) BSA, 1mM EDTA, 1mM $MnCl_2$, 400µg/ml bacitracin, 20µg/ml leupeptin and 1mM Pefabloc™ SC. The assay plate was counted using the EG&G Wallac 1450 MicroBeta™ scintillation counter.

For 384-well FP assays, CHO NK_1 cell membranes (~1pmol/mg) were incubated with either of the Cy3B ligands (0.5nM) in the presence of varying amounts of Sub P, [Sar^{9}Met$(O_2)^{11}$] Sub P or Neurokinin-A (NKA) for 3.5 hours at room temperature (20 – 25°C) in 50mM Tris buffer pH7.5 containing 0.5%(w/v) BSA, 1mM EDTA, 1mM $MnCl_2$, 400µg/ml bacitracin, 20µg/ml leupeptin and 1mM Pefabloc SC. Incubations were performed in a total volume of 50µl in Corning black 384-well non-binding surface plates. Non-specific binding (NSB) was determined in the presence of 25µM Sub P. FP was then determined on FARCyte using a Cy3B optical filter/dichroic configuration. Specific polarization values were calculated by subtracting the polarization signal obtained in NSB wells (in the presence of 25µM Sub P) from the test well values.
Further assays were performed as described with Cy3B-(terminal)-Sub P (0.5nM) only, in a total volume of 8µl in Greiner black 1536-well microtitre plates.

Results

Maximal FP change in the competitive displacement assay signal was observed using Cy3B-(terminal)-Sub P but consistent results were obtained with both ligands (Figure 1). Similar performance using Cy3B-(terminal)-Sub P was also observed during further miniaturization of the assay to a 1536-well format (Figure 2).

Figure 1: NK_1 competitive binding assays (Sub P, [Sar^{9}Met$(O_2)^{11}$] Sub P and NKA) in 384-well format.

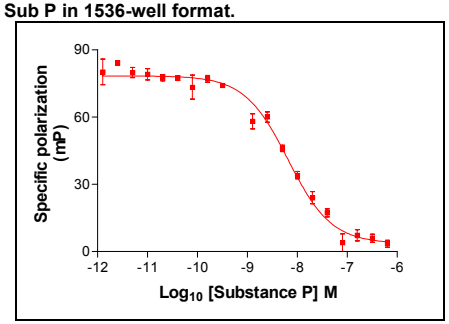


CHO NK_1 cell membranes (30µg) were incubated with a) 0.5nM Cy3B-(terminal)-Sub P or b) 0.5nM Cy3B-(lysine)-Sub P in the presence of Sub P (■), [Sar^{9}Met$(O_2)^{11}$]-Sub P (▲) or NKA (▼) as described in the text and FP was measured on FARCyte. Values are plotted as means of quadruplicates ± SEM.

Z' factor analysis, recently described by Zhang *et al* [1], describes assays with a Z' factor between 0.5 and 1.0 to be reliable and robust for HTS. Here values of 0.68 and 0.51 were obtained for the 384-well and 1536-well formats respectively (Figure 3), indicating both are suitable for HTS purposes.

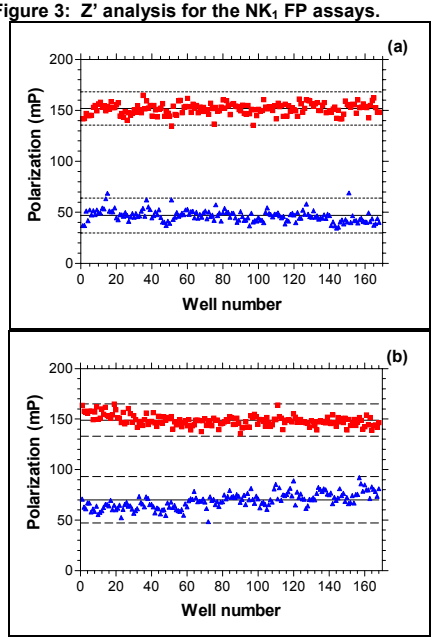
Table 2 compares both the 384-well and 1536-well assays for the NK_1 receptor. Miniaturization represented an approximate 6-fold reduction in membrane/ligand usage and assay volume. In addition, the ability to monitor competitive displacement and observe acceptable assay reliability was retained.

Figure 2: NK_1 competitive binding assay with Sub P in 1536-well format.



CHO NK_1 cell membranes (5µg) were incubated with 0.5nM Cy3B-(terminal)-Sub P and varying concentrations of Sub P as described in the text and FP was subsequently measured on FARCyte. Values are plotted as means of quadruplicates ± SEM.

Figure 3: Z' analysis for the NK_1 FP assays.



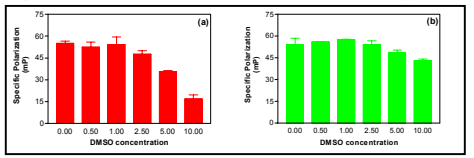
CHO NK_1 cell membranes (a) 30µg in 384-well plates and (b) 5µg in 1536-well plates were incubated with 0.5nM Cy3B-(terminal)- Sub P in the absence (■) or the presence (▲) of 25µM Sub P as described in the text and FP measured on 168 replicates of each on FARCyte.

In order to assess the applicability of such assays to HTS environments, the effect of DMSO was investigated. The assay tolerated up to 2.5% (v/v) DMSO. At higher concentrations a 70% reduction in the binding assay signal was observed (Figure 4a). We conclude this to be an effect on binding efficacy in this assay as when DMSO was added after the completion of binding, signal was reduced by only 20% even in the presence of 10% DMSO (Figure 4b).

Table 2: Summary of FP assay miniaturization.

Assay conditions	*384-well*	*1536-well*	*Reduction (fold)*
Membrane protein	*30µg*	*5µg*	*6*
Cy3B-(terminal)-Sub P	*25fmol*	*4fmol*	*6.25*
Volume of the assay	*50µl*	*8µl*	*6.25*
Final Conc. of Cy3B-(terminal)-Sub P	*0.5nM*	*0.5nM*	*-*
Apparent IC_{50} (Sub P)	*4.3nM*	*6.5nM*	*-*
Assay Z' factor	*0.68*	*0.51*	*-*

Figure 4: Effect of DMSO on the NK_1 FP assay.



CHO NK1 membranes (30µg) were incubated with 0.5nM Cy3B-(terminal)-Sub P as described in the text together with varying concentrations (v/v) of DMSO (0.5, 1, 2.5, 5 or 10%) either (a) from the outset (■) or (b) following completion (■) of the binding incubation. FP was subsequently measured on FARCyte. Values are plotted as means of quadruplicates ± SEM.

CONCLUSIONS

- **We have previously miniaturized a small molecule FP receptor ligand assay[2] and here have successfully miniaturized an FP receptor peptide ligand assay in 384 and 1536-well formats using a Cy3B label and FARCyte.**
- **The miniaturization significantly reduced reagent consumption and assay volume without compromising assay reliability.**
- **The apparent IC_{50} values obtained in both formats were consistent and Z' values of >0.5 were also observed.**
- **These assays have used common receptor expression levels (~1pmol/mg) and low ligand concentrations (0.5nM), extending the potential of this technique to other small molecule or peptide ligand binding assays in HTS.**

References:

1. Zhang, J-H., *et al* (1999), *J. Biomol. Screening*, **2**, 67 - 73.
2. Harris, A.J., *et al* (2001), poster presentation at "Drug Discovery Technologies", Boston, MA,

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