THE USE OF FLUORESCENCE TECHNOLOGIES TO MEASURE SPECIFIC SIGNAL TRANSDUCTION EVENTS AND MOLECULAR INTERACTIONS

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Introduction

Signal transduction events and molecular interactions are popular targets for drug discovery by pharmaceutical companies. Radioactive methodologies are often employed; scintillation proximity asssay (SPA) is an example of a versatile homogeneous radioactive screeening technology that is widely used by major pharmaceutical companies[1]. Increasingly, there is a desire to move towards non-radioactive technologies, particularly fluorescence[2].

NF-κB is a transcription factor of potential therapeutic relevance due to its central role in regulating the transcription of a large number of genes involved in cellular defence[3]. In this study we have investigated the interaction of the p65 subunit to a dsDNA substrate (shown below) containing the 10bp consensus DNA binding sequence (underlined) to which it binds with high affinity[4].

5'-dye-GATCTA**GGGACTTTCC**GCC
ATC**CCCTGAAAGG**CGCCTAG-3'

Grb2 is an adaptor protein containing one SH2 domain flanked by two SH3 domains; the SH2 domain binds to phosphotyrosine containing sequences on the C-terminal ends of epidermal growth factor (EGF) receptor and other receptors. Peptides based on the sequence around pY-1068 of EGF receptor were selected[5](see Table 1).

Table 1:Peptide sequences for Grb2 binding assays.

Code	Sequence
136P	AcLPVPEpYINQSVPK
136	AcLPVPEYINQSVPK
V11K	VPEpYINQSVPK
P10K	PEpYINQSVPK
E9K	EpYINQSVPK

These two model systems have been used to discuss and compare two homogeneous fluorescence technologies, fluorescence polarisation[6] and fluorescence energy transfer (FRET), one heterogeneous format and the potential utility of the near IR fluorophores Cy™3 and Cy5 for these techniques.

Results

Protein-DNA

The dsDNA sequence was labelled with both fluorescein and Cy5.
The polarisation study in Figure 1a shows the interaction between labelled dsDNA and increasing concentrations of NF-κB p65 protein. The K_D values determined by non-linear regression (one binding site hyperbola) analysis for fluorescein dsDNA and Cy5 dsDNA were 27nM and 24nM respectively; these values are considerably lower than values reported for similar dsDNA sequences (typically pM) using SPA (data not shown) or reported in the literature[4]. This observed reduction in affinity may well be due to effects of labelling the dsDNA with the fluors; further work is ongoing. Also, competition binding in the presence of specific dsDNA was demonstrated (see Figure 2a).

Figure 1. Interaction of dye labelled dsDNA with NF-κB p65 protein measured using fluorescence polarisation and FRET.

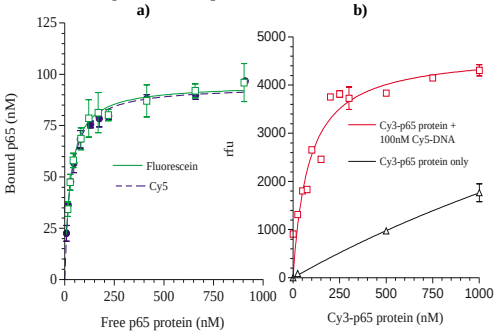

Figure 1. a) *For the polarisation assay, dye labelled dsDNA (100nM) was incubated in assay buffer (10nM HEPES pH 7.0 containing 0.2mM EDTA, 20mM NaOAC, 0.005% IPEGAL and 5mM DTT) with various concentrations of NF-κB p65 protein in a final volume of 100µl in black 96-well microplates, and read in a Fluorolite FPM-2™ microplate reader.* ***b)*** *In the FRET format, Cy5 labelled dsDNA (100nM) was incubated in the same assay buffer plus 0.1%BSA with various concentrations of Cy3 labelled NF-κB p65 protein and read in a Biolumin™ 960 fluorescence microplate reader.*

FRET is a homogeneous format, but the labelling of both binding components with retention of biological activity is required. The cyanine dyes Cy3 and Cy5 are a pair of dyes which are suitable as donor and acceptor respectively. A successful FRET assay could be demonstrated by labelling the dsDNA with Cy5 and the NF-κB p65 protein with Cy3. Saturation with increasing concentrations of Cy3 labelled NF−κB was demonstrated (see figure 1b).

Figure 2. Competition polarisation and FRET assays with unlabelled specific dsDNA for the NF−κB protein-DNA interaction.

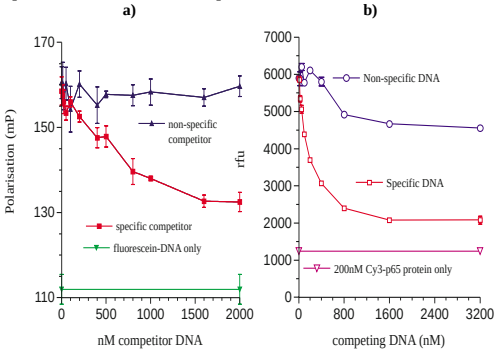

Figure 2. a) *For the polarisation assay, fluorescein labelled dsDNA (100nM) and NF-κB p65 protein (300nM) were incubated with various concentrations of dsDNA (specific and non-specific)* ***b)*** *For the FRET assay, Cy5 labelled dsDNA (100nM) and Cy3 labelled NF-κB p65 protein (200nM) were incubated with dsDNA. Other conditions were as described in Figure 1.*

Competition binding with unlabelled specific dsDNA similar to that for the polarisation format was demonstrated; unlabelled non-specific dsDNA had little effect (see figure 2b). Competition was also observed in the presence of unlabelled NF-κB p65 protein (data not shown). Energy transfer was not observed when Cy3 labelled dsDNA and Cy5 labelled NF-κB p65 protein were used ; the reason for this is not clear and is being further investigated.

It is possible to set up a heterogeneous separation format for this interaction. The same dsDNA sequence as used previously was labelled with biotin and bound to streptavidin coated 96-well microplates. After interaction with NF-κB p65 glutathione-S-transferase (GST) fusion protein and washing, detection was with Cy3 or Cy5 labelled anti-GST antibody. Data for various concentrations of NF−κB p65 GST fusion protein are shown in Figure 3. Good signal to noise ratios are obtained, typically 50:1 for detection with Cy5 and 100+:1 for detection with Cy3. This format requires fluorescent labelling of a generic antibody reagent rather than the NF- κB p65 GST fusion protein.

Figure 3. Heterogeneous separation format assay for NF-κB p65 GST fusion protein-DNA interaction.

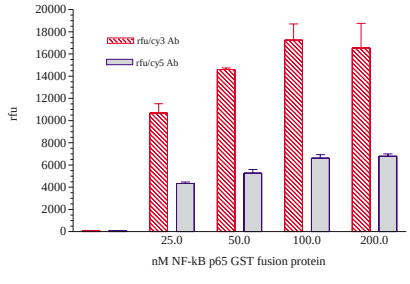

Figure 3. *2.5pmoles of biotin labelled dsDNA was added to each well of a 96-well streptavidin coated black microplate. After washing, various concentrations of NF-κB p65 GST fusion protein were added in assay buffer (10nM HEPES pH 7.0 containing 0.2mM EDTA, 20mM NaOAC, 0.005% IPEGAL, 0.1%BSA and 5mM DTT) in a final volume of 100µl with various concentrations of NF-κB p65 protein and incubated at room temperature. Detection was with 50pmoles of Cy3 or Cy5 labelled anti-GST antibody per well. The plates were read in a Biolumin 960 fluorescence microplate reader.*

Protein-peptide

For a fluorescence polarisation based assay, peptides E9K, P10K and V11K were labelled at their N-termini with fluorescein to give F-E9K, F-P10K and F-V11K respectively. Retention of binding activity upon fluorescein labelling was confirmed in a SPA binding assay using [^{3}H]136P as ligand (data not shown). The affinity of peptide 136P for the Grb2-GST fusion protein was found to be 450nM by SPA (data not shown).

In their polarisation study using the STAT1 SH2 domain, Wu *et al.*[7] observed that the fluorescent probe should not be separated from the phosphotyrosine residue by more than one amino acid residue to observe an acceptable change in polarisation. However using F-E9K, F-P10K or F-V11K as ligand, an approximately 30mP change in polarisation could be observed using 1.1µg of Grb2 protein per well and 20nM fluorescent tracer. SPA data would suggest that 10% of the added tracer is being bound using 1.1µg Grb2 protein per well, thereforea 30 mP change in polarisation is reasonable; larger changes an be observed using more Grb2 protein.

Competition curves are shown in Figure 4a. In agreement with SPA data, peptide E9K is a slightly less effective competitor than peptide 136P; the non-phosphorylated peptide 136 does not compete at all. The assay was also set up using the near IR fluoroprobe Cy5; saturation curves using both F-P10K and Cy5 labelled E9K are shoew in Figure 4b.

Useful FRET assays using Cy3 as donor and Cy5 as acceptor could not be established, even though both the peptide and Grb2 protein could be labelled with good retention of binding activity in the SPA format assay.

Figure 4. Polarisation assays for the Grb2 SH2 domain binding interaction.

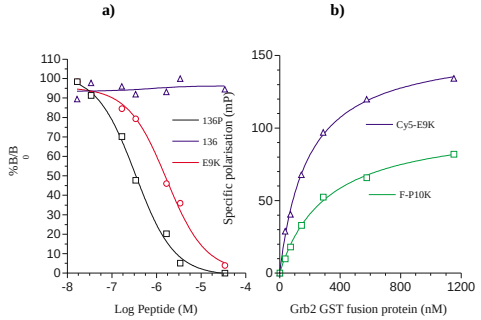

Figure 4. a) *1.1µg Grb2 GST fusion protein per well, various concentrations of competing peptide and 20nM F-P10K in 20mM MOPS pH7.4/10nM DTT/ 0.005% Tween 20 were incubated in a final volume of 150µl in black 96-well microplates, and read in a Fluorolite FPM-2 microplate reader.* ***b)*** *20nM F-P10K or Cy5-E9K were incubated with various concentrations of Grb2 GST fusion protein; 100µM peptide 136P was used to define non-specific binding.*

Conclusions

1. Fluorescence technologies have the potential to be applied to the measurement of signal transduction events.

2. For the protein-DNA interaction model system, homogeneous polarisation and FRET format assays have been developed, but there may be effects on affinity. Also, a heterogeneous separation format 96-well microplate assay has been demonstrated.

3. For the lower affinity protein-peptide model system, a polarisation format assay was developed. Labelling with the fluorescent probe did not significantly affect the binding activity of the peptide. It was not possible to establish a FRET based assay.

4. In the development of fluorescent based assays, careful attention has to be paid to the labelling of molecules with the fluorescent probes to maintain binding activity.

References

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