Assay and array technologies for G-protein coupled receptors

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1 Introduction

1.1 How and why: investigating G-protein coupled receptors as recognition molecules in biosensors and biochips?

G-protein coupled receptors (GPCRs) are cell surface receptors located within the lipid bilayer of the cell. They are the largest family of membrane proteins in the human genome and are involved in a number of cell signalling pathways within the cell. This thesis investigates the methods by which signalling via these molecules can be monitored in the absence of the cell, in a format adaptable to a biochip or biosensing platform.

Biosensors are, in general, analytical devices based on a biologically active compound coupled to a signal transduction mechanism (Malhotra et al. 2005). The interaction of analyte with the biomolecule is designed to produce an effect which can be measured by the transducer. Protein immobilization (Rusmini, Zhong & Feijen 2007) or entrapment strategies (Brennan 2007, Lin, Wu & Brennan 2007, Shen et al. 2007) and signal transduction techniques (Marazuela & Moreno-Bondi 2002, Homola, Yee & Gauglitz 1999, Bunde, Jarvi & Rosentreter 1998, Thevenot et al. 2001, Grieshaber et al. 2008, Jaffrezic-Renault & Dzyadevych 2008, Ramanathan & Danielsson 2001) are important in biosensor development. The term “biochips” generally applies to an array of individual biosensors, and can also be referred to as DNA or protein microarrays. Protein arrays provide a platform by which analysis of gene products at the functional level becomes possible. In particular, interest lies in the arraying of membrane proteins (e.g. GPCRs), due to their high importance in therapeutics and therefore in the discovery of new drug compounds (see section 1.4). Further to this, the incorporation of early signalling events triggered by activation of these receptors into the biochip or sensor platform, could provide direct evidence of the function of the binding ligand, be it an activator or inhibitor of receptor activity. This thesis investigates methods for the expression, assay and array of G-protein coupled receptors and their associated signalling molecules, the G-proteins.
Signal transduction within the cell by G-protein coupled receptors begins with the binding of a specific ligand to the receptor binding pocket. Following this, the receptor induces conformational changes in the associated messenger proteins known as the G-proteins. These three dissimilar proteins $G\alpha$, $G\beta$ and $G\gamma$ exist together as a heterotrimer until activated by the receptor, at which time a nucleotide exchange occurs on the $G\alpha$ subunit, and the heterotrimer has been shown in vitro, to separate (Leifert et al. 2005b) (for further discussion see section 1.3.1.1). GPCR signal transduction can be monitored both within the cell (cell-based) and in isolated membrane fragments or reconstituted lipid bilayers (cell-free) formats. Both strategies are capable of miniaturization and amenable to high throughput screening (HTS), however, cell-based methodologies, whilst useful for obtaining valuable information regarding biological response, can be problematic in HTS due to increased complexities in their implementation as well as assay variation (Maddox, Rasmussen & Lucile 2008). Cell-free assay methodologies, have in the past, primarily relied on radioactive ligands and nucleotides for use in filtration based assays, a format which is not particularly adaptable to high-throughput screening. Fluorescence techniques such as fluorescence polarization and fluorescence resonance energy transfer (FRET) (for more details on the FRET technique, see section 3.1.3) have provided an alternative to the use of radioactive isotopes and enabled the production of homogeneous (mix-and-read) assay formats as opposed to separation/filtration assays, thereby increasing the speed and portability of the assay design. This thesis discusses the strategy by which a fluorescence-based assay (utilizing time-resolved (TR)-FRET) could be used to monitor receptor induced activation by monitoring the separation of the G-protein subunits (see chapter 3). The TR-FRET system could be utilized in well-based assays (using microtiter plates) or possibly in array-based formats displaying the GPCR signalling system.

In order to investigate the viability of creating a device loaded with this GPCR recognition/transduction element for biochip or biosensing applications, this thesis goes on to investigate a strategy to immobilise the proteins onto a surface. The immobilization strategy utilises the high affinity interaction of complementary oligonucleotides to capture native lipid vesicles containing GPCRs and potentially the whole G-protein signalling assembly (GPCR + G-proteins). The advantage of the oligonucleotide based system includes the potential of the protein embedded vesicles to
self-sort onto an array platform, possibly leading to a production methodology for a membrane protein array composed of a number of different receptors.

Possible limitations in using native vesicles to produce a durable device or chip, is potentially the lack of vesicle stability. An alternative membrane protein array platform is further investigated within the scope of this thesis as a means of addressing this. Particles are introduced, which are composed of amphiphilic molecules that could possibly be applicable as a substitute microenvironment for the lipid vesicles. Instead of the unilamellar enclosed bilayer of the vesicles, these suspensions exist in the cubic phase, as particles known as cubosomes. This lipid or surfactant cubic phase environment is commonly used for membrane proteins in crystallization trials (Caffrey 2000). These cubosome particles are investigated for their adaptability to the site-directed oligonucleotide immobilisation technique as was used with the GPCR containing vesicles. It has been indicated that cubosomes are more stable than liposomes or vesicles due to their higher bilayer area to particle volume ratio (Garg, Saraf & Saraf 2007), as well as the inclusion of stabiliser molecules in the porous cubosome structure, to eliminate aggregation. This stability could be useful in the future establishment of membrane protein arrays.

1.2 G-protein coupled receptors

In order to express functional tertiary structures of GPCRs and subsequently remove them from their whole-cell environment, it is important to understand the structure, function and environmental requirements of these membrane proteins.

1.2.1 Structure and function

GPCRs are generally classified into groups (termed, class A-F) (Kolakowski 1994), with the class A (rhodopsin-like) family being the largest, and the members of which are investigated in this thesis. The general architecture (as constructed from amino acid sequences and modelling) of the class A GPCRs, most commonly include an extracellular amino-terminus (N-terminus), seven-membrane-spanning α-helices connected by 3 intracellular and 3 extracellular loops, and an intracellular carboxy-terminus (C-terminus). As the name suggests, the receptors couple to intracellular proteins, the G-proteins at the C-terminus, which upon activation initiate various
signalling events within the cell. Signalling transduction is generally dependent on the family of the coupled G-protein (Figure 1.2.1). While many receptors couple to a specific G-protein subtype, some receptor types have the capability of activating multiple G-proteins e.g. a single light activated rhodopsin can activate multiple $G_i$ subunits (Arshavsky, Lamb & Pugh 2002). Suggestions have also been made in regards to ligand directed G-protein coupling, as it has been observed that different agonists activating the same receptor can result in different G-protein signalling pathways being activated (Kenakin 2003).

![Figure 1.2.1 Schematic representing membrane spanning GPCR and coupled G-protein heterotrimer.](image)

The GPCR transmembrane domains share a high degree of sequence similarity between receptor subtypes of the same family, but the intra- and extracellular domains exhibit variability in size and sequence. Co- and/or post-translational modifications of the receptors are implicated in their function. Many GPCRs have one or more sites for N-glycosylation (involved in GPCR trafficking within the cell) (Duvernay, Filipeanu & Wu 2005), and some Cys residues have been known to become palmitoylated (covalent attachment of fatty acids), a modification which has been suggested to play a role in controlling the interaction of GPCRs with specific regulatory proteins (Escriba et al. 2007). Additionally, orientation of the extracellular loops (which can be involved in ligand binding) in class A GPCRs, are maintained by a crucial disulfide bond between extracellular loops 1 and 2 (McCusker et al. 2007).
Interaction sites between receptor and ligand and receptor and G-proteins are important to consider when designing labelling strategies for the protein complex. The binding of an agonist to the extracellular domains (or transmembrane) sites of a GPCR produces a conformational change in the receptor, specifically, the receptor demonstrates an outward movement of helix VI away from the receptor core and towards the membrane, as was shown for the β2-adrenergic receptor (Jensen et al. 2001) and the M₃-muscarinic receptor (Ward et al. 2006). This conformational change is transmitted to intracellular domains in contact with the G-proteins. The C-terminus of the Gα subunit and the intracellular loops (most commonly the 2nd and 3rd loop, but it is dependent on receptor type) are implicated in the GPCR/G-protein binding interface (Oldham & Hamm 2008).

The active and inactive complexation activity between GPCR and G-protein is important to consider when using assays whereby the location of one protein in relation to another is the means by which functional activity is measured, such as resonance energy transfer methods (discussed more in chapter 2). There are two main models to explain receptor complexation during activation. One is the ‘collisional coupling’ (or extended ternary) model which illustrates G-protein movement along the plasma membrane and coupling, upon interaction, to activated receptors. The second is the cubic ternary model (Figure 1.2.2), which suggests that the G-proteins can interact with receptors before agonist (a ligand which results in the activation of the receptor) binding (that is, they can pre-couple to the receptor) (Weiss et al. 1996).
Figure 1.2.2 Pharmacological model for G-protein coupled receptor activation: The GPCR can exist in two states, active (R*) and inactive (R). Both the ligand (L) and G-protein (G) can couple to the active or inactive form of the receptor. This gives eight possible complex states in which the receptor can reside: R; R*; LR; LR*; LRG; LR*G; RG; R*G. This is the cubic ternary complex model described by Weiss et al. (Weiss et al. 1996).

One such example of this complex pre-coupling was shown using FRET (for more details on this technique see section 3.1.3), between a fluorescently tagged α2-adrenergic receptor and a fluorescently tagged Gα1β1γ2 heterotrimer in the absence of agonist, suggesting a pre-formed receptor-G-protein complex (Hein et al. 2005). Additionally, in vitro studies involving the δ-opioid receptor and Gα1, have demonstrated a high affinity binding interaction between the ligand-free receptor and the G-protein subunit (~60 nM) using plasmon waveguide spectroscopy (Alves et al. 2003). This study showed the highest affinity interaction occurred between an agonist bound receptor and the G-protein (~10 nM), while the lowest affinity interaction occurred between the antagonist bound receptor and the G-protein (~500 nM) and no interaction was observed between the two when an inverse agonist was bound to the receptor (for more information about ligand classification see section 1.2.1.1). This model of a pre-formed complex between the receptor and G-proteins indicates that these proteins can be captured together in the inactive form on a sensor or array surface, and changes in complex state can be an indicator of activation induced by a test compound.

GPCR activity has also been shown to involve multiple receptors (dimerisation and oligomerisation) (Rios et al. 2001, Carrillo, Stevens & Milligan 2002, Milligan et al. 2003, Dalrymple, Pfleger & Eidne 2008, Gurevich & Gurevich 2008b, Gurevich &
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Gurevich 2008a, Milligan 2005, Szidonya, Cserzo & Hunyady 2008). Although not investigated in this thesis, it is an example why receptor freedom of movement within the lipid bilayer is important. The strategy of immobilization within a vesicle or other lipid-based carrier, is designed to maximise receptor freedom and accessibility (for more details see chapter 4). Additionally, dimerization and/or oligomerization is another mechanism of the GPCR signalling process (other than the G-protein interaction) which could potentially be investigated using the TR-FRET methods described in this thesis.

Within the cell, GPCR deactivation and downregulation after ligand activation is modulated by G-protein coupled receptor kinases (GRKs) (receptor phosphorylation) and β-arrestins (G-protein uncoupling). Modulator proteins (e.g. β-arrestin (Ross et al. 2008) ), have been used in the design of GPCR assay systems and could also be considered for use in an array or sensing device where the signalling components are entrapped or embedded together on a surface. For the ability to monitor receptor/protein interactions such as these in vitro, full accessibility and mobility of receptors is important, as alluded to with receptor/receptor interactions above.

1.2.1.1 GPCR ligands

GPCRs have an extensive range of ligands including light, odorants/ volatiles, neurotransmitters and hormones (see Figure 1.2.1). The effect each ligand has on the receptor once bound depends on the classification of that ligand. Ligand classification is based on two important properties, 1) the affinity of the ligand and its receptor, and 2) the efficacy of the ligand, i.e. once bound to the receptor, the ability of the ligand to produce a response. Traditionally, ligands were classified as agonists (ligands with affinity and efficacy for a particular receptor) and antagonists (ligands with affinity for a particular receptor). These were described to act on GPCRs by activating G-protein signalling (agonist), and blocking the receptor binding site (antagonist). This classification has now evolved to include full agonists, partial agonists, neutral antagonists and inverse agonists (Urban et al. 2007). Full agonists produce maximal stimulation of cellular response linked to a particular receptor, whereas partial agonists produce a submaximal response. Inverse agonists have been described as ligands which induce a negative efficacy on the receptor it has affinity for, i.e. they reduce constitutive activity (ligand-independent, basal receptor signalling). It has been suggested that up to
85% of competitive antagonists, are in fact inverse agonists (Kenakin 2004). The neutral antagonist possesses no intrinsic efficacy, but blocks the receptor binding site to inhibit the effects of agonists. It is also worth mentioning that there are allosteric modulators which can effect GPCR functional activity when they bind to alternative (allosteric site) binding sites rather than the main agonist/antagonist binding site (orthosteric site) on the receptor (Christopoulos & Kenakin 2002, Christopoulos et al. 2004, May et al. 2007). Developments of methods to screen compounds for their allosteric activity of GPCRs in a high-throughput manner would be desirable.

1.3 G-proteins

1.3.1 Structure and function

The G-proteins are important components of the signalling cascade initiated by GPCR activation. It is the proximity and relationship of the three protein subunits (G$_\alpha$, G$_\beta$ and G$_\gamma$), to each other at different stages of activation, that is of interest as a means to measure the activation state of the receptor to which they are coupled.

The structures of the G-protein subunits are important to consider prior to modification via the attachment of fluorescent probes; a strategy employed in this thesis. The G$_\alpha$ subunit is composed of two domains; a GTPase domain and a helical domain consisting of $\alpha$ helices and connecting loops (Remmers et al. 1999). The core of the G$_\beta$ subunit folds into a toroidal $\beta$-propeller domain composed of what is termed $\beta$-blades (Wall et al. 1995), while the neighbouring G$_\gamma_2$ forms a helical structure. The G$_\beta$ and G$_\gamma$ subunits exist as a tightly bound dimer and can be resolved only in the presence of denaturants (Wall et al. 1995). The crystallographic structure of the G-protein heterotrimer (G$_{\alpha_i}\beta_1\gamma_2$) reveals two regions of contact between the $\alpha$ and $\beta$ subunits (Wall et al. 1995).

The major $\alpha/\beta$ interface covers switch II of $\alpha$ (a region implicated in GTPase activity (Wall, Posner & Sprang 1998)) while the second site of contact is between the helical N-terminus of $\alpha_i$ and the side of the $\beta$ propeller in the $\beta_1$ subunit. The interface between $\beta_1$ and the $\gamma_2$ subunit is extensive with the $\gamma_2$ subunit ultimately embedded within $\beta_1$. 
The G-protein heterotrimer undergoes both co- and post-translational lipid additions (Vogler et al. 2008). The Gα subunits undergo N-myristoylation and/or palmitoylation. Myristoylation represents the attachment of myristate through an amide bond at a glycine residue at the N-terminus. Palmitoylation refers to the attachment of palmitate through a thioester bond to a cysteine residue near the N-terminus. (Chen & Manning 2001). Additionally, the Gγ subunit of the Gβγ dimer is also modified with a lipid addition, this time at the C-terminus (at the CAAX motif). This addition is referred to as isoprenylation, and differs between subunit types as either a geranylgeranyl moiety or a farnesyl moiety (Clapham & Neer 1997). These lipid modifications aid in membrane targeting, protein interactions and mobilization of proteins to different membrane microdomains (specialized lipid domains within the plasma membrane) (Vogler et al. 2008).

1.3.1.1 G-protein activation cycle

The G-protein heterotrimer activation cycle is triggered when the GPCR is activated by its cognate ligand and is schematically represented in Figure 1.3.1. The G-protein cycle is initiated by a conformational change in the Gα subunit, which is responsible for the exchange of GTP for GDP in the guanosine nucleotide binding domain on the Gα subunit. Remmer et al. (1999) have implicated the amino acid composition of C-terminal helix of the Gα subunit as a critical component in the rate of GDP release (a process integral to the nucleotide exchange) (Remmers et al. 1999). Binding of GTP and Mg2+ activates the Gα subunit which is then proposed to dissociate from the βγ dimer to interact with downstream effectors. The intrinsic GTPase activity of the Gα subunit hydrolyses the bound GTP, providing a mechanism to terminate the activation signal. The heterotrimer is then believed to reform in the inactive GDP bound state.
Figure 1.3.1 Schematic representing the activation cycle of the G-protein heterotrimer. Upon agonist activation the receptor (R) undergoes a conformational change (R*) which promotes the exchange of GTP for GDP on the Ga subunit. The conformational change between the Ga and Gbγ subunits exposes effector (E) binding sites. The active form of the G-proteins activate these effectors (E*) for further downstream signalling. The intrinsic GTPase activity of the Ga subunit terminates the signal via dephosphorylation of the bound GTP to GDP and the subsequent reformation of the inactive GDP-bound heterotrimer. (McMurchie & Leifert 2006)

This dissociation/association cycle continues to be investigated by various research groups because there has been evidence of heterotrimer rearrangement rather than complete dissociation upon activation. Bunemann et al. have demonstrated, using a cell-based FRET approach, an increase in FRET induced fluorescence upon GPCR activation (Bunemann, Frank & Lohse 2003). This increase is observed when Yellow Fluorescent Protein (YFP) - and Cyan Fluorescent Protein (CFP) - fluorescent fusion proteins are expressed in the α-helical domain of the Ga and the N-terminus of the β subunit respectively, opposing the common notion that the subunits dissociate upon activation (which would have resulted in a decrease in FRET). Another study using a similar technique reported different results for different sets of G-protein subunits. Results from FRET based studies by Frank et al. using Gαo and Gαi1 subunits exhibited decreases and increases in FRET induced fluorescence respectively (Frank et al. 2005). Changes between Gαo-YFP and βγ-CFP upon activation were indicative of the rearrangement (or dissociation for the Gαo subunit) of the activated Gα subunit and dependent on the Gα subtype being monitored. The fluorescent proteins used in the FRET studies mentioned above are quite large (GFP ~30kD), and approximately the same size as the G-proteins themselves. Modification of the G-proteins with such a large fusion protein could potentially restrict or alter the movement of the subunits (for more detailed discussion of protein labelling see section 3.1.2). In this study, small
molecular weight probes will be used to eliminate potential problems associated with large fusion proteins. In addition, a specific probe with a long emission lifetime (lanthanide) is used to increase the signal over noise in the system (see section 3.1.4.1). Recently, in vitro investigations have provided further evidence for subunit separation upon GPCR activation (Leifert et al. 2005b). Additionally, Digby et al. demonstrated that at least some G-protein subtypes do separate within the cell (Digby et al. 2006). Regardless of whether the G-proteins separate or rearrange, the important result would be the visualization of a change in G-protein conformation and therefore a measurable change in fluorescence upon ligand induced receptor activation.

1.4 GPCRs and G-proteins: potential for therapeutics

The importance and effectiveness of drugs that target GPCRs are evident by their prominence amongst the top-selling prescription drugs on the market (see Table 1.3.1-1). There are reported to be approximately 747 different human GPCRs (Vassilatis et al. 2003) with only approximately 230 having known natural ligands (non-odorant ligands) and only about 30 receptor types commonly targeted for therapeutic purposes (Klabunde & Hessler 2002). The receptors for which the ligand and the physiological function is unknown are known as “orphan receptors” (Wise, Gearing & Rees 2002), and through the characterisation of these receptors, many new opportunities for developing novel therapeutics could emerge.
Table 1.3.1-1: Examples of prescription drugs which target GPCRs for the indicated disease state
(McMurchie & Leifert 2006)

<table>
<thead>
<tr>
<th>Brand Name</th>
<th>Generic Name</th>
<th>G-protein coupled receptor(s)</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zyprexa</td>
<td>Olanzapine</td>
<td>Serotonin 5-HT2 and Dopamine</td>
<td>Schizophrenia, Antipsychotic</td>
</tr>
<tr>
<td>Risperdal</td>
<td>Risperidone</td>
<td>Serotonin 5-HT2</td>
<td>Schizophrenia</td>
</tr>
<tr>
<td>Claritin</td>
<td>Loratidine</td>
<td>Histamine H1</td>
<td>Rhinitis, Allergies</td>
</tr>
<tr>
<td>Imigran</td>
<td>Sumatriptan</td>
<td>Serotonin 5-HT1B/1D</td>
<td>Migraine</td>
</tr>
<tr>
<td>Cardura</td>
<td>Doxazosin</td>
<td>α-adrenoceptor</td>
<td>Prostate hypertrophy</td>
</tr>
<tr>
<td>Tenormin</td>
<td>Atenolol</td>
<td>β1-adrenoceptor</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>Serevent</td>
<td>Salmeterol</td>
<td>β2-adrenoceptor</td>
<td>Asthma</td>
</tr>
<tr>
<td>Duragesic</td>
<td>Fentanyl</td>
<td>Opioid</td>
<td>Pain</td>
</tr>
<tr>
<td>Imodium</td>
<td>Loperamide</td>
<td>Opioid</td>
<td>Diarrhoea</td>
</tr>
<tr>
<td>Cozaar</td>
<td>Losartan</td>
<td>Angiotensin II</td>
<td>Hypertension</td>
</tr>
<tr>
<td>Zantac</td>
<td>Ranitidine</td>
<td>Histamine H2</td>
<td>Peptic ulcer</td>
</tr>
<tr>
<td>Cytotec</td>
<td>Misoprostol</td>
<td>Prostaglandin PGE1</td>
<td>Ulcer</td>
</tr>
<tr>
<td>Zoladex</td>
<td>Goserelin</td>
<td>Gonadotrophin-releasing factor</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>Requip</td>
<td>Ropinirole</td>
<td>Dopamine</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>Atrovent</td>
<td>Ipratropium</td>
<td>Muscarinic</td>
<td>Chronic obstructive pulmonary disease (COPD)</td>
</tr>
</tbody>
</table>

Structure-based drug design approaches for GPCR targets has in the past been supported by the first characterization of a three-dimensional crystal of a GPCR, which was the visual photoreceptor protein, rhodopsin (Palczewski et al. 2000). Although revealing only low sequence similarity to other GPCRs, rhodopsin represents an improved structural template for both the understanding of experimental data available for other seven-transmembrane receptors and for generating improved molecular models for other receptors of similar structure (Klabunde & Hessler 2002). These studies have helped determine which amino acids play a key role in the interaction between receptor and ligand. Advances in receptor purification, crystallization and crystal diffraction protocols have led to the recent elucidation of the β2-adrenergic receptor (Rasmussen et al. 2007, Warne et al. 2008, Park et al. 2008), the β1-adrenergic receptor (Warne et al. 2008) and the Opsin receptor (Park et al. 2008), which is likely to be the beginning of a flood of GPCR structural data.

Suitable molecular modelling programs are now available to screen and recognize biologically active sites within known GPCRs and cognate ligands through sophisticated docking routines (Klabunde & Hessler 2002). The syntheses of
combinatorial libraries (Beno & Mason 2001) have also been developed to identify new compounds that could potentially act on various GPCRs. The need to screen these compounds to test their validity as GPCR specific ligands necessitates the development of GPCR based assays with particular emphasis on increasing assay throughput.

Studies have also established the potential of the intracellular machinery, including the Receptor-G-protein interface (Gilchrist et al. 1998) and the heterotrimeric G-protein subunits as drug targets (Hessling, Lohse & Klotz 2003, Holler, Freissmuth & Nanoff 1999) as opposed to exclusively targeting cell surface receptor proteins per se. It has been suggested that ligands directly targeted to G-proteins could potentially modulate individual effector pathways, alter signals specifically from particular G-protein classes or subclasses and/or modify the kinetics of G-protein signalling (Ja & Roberts 2005). It can be envisaged that a G-protein based microarray capable of showing interactions of G-proteins both in the presence and absence of a GPCR would have applications in screening potential therapeutic compounds.

1.5 Current assay technologies for monitoring G-protein coupled receptors

1.5.1 Cell-based assays

Methods used to measure G-protein coupled receptor activation traditionally involve cultured cells transfected with the GPCR and G-proteins of interest. Ligand activation of GPCRs can be monitored using intracellular second messengers such as 3'-5'-cyclic adenosine monophosphate (cAMP), calcium, inositol phosphatases or extracellular signal-regulated protein kinase 1/2 (ERK 1/2). Intracellular messengers can be measured in a variety of ways including the use of fluorescent compounds, e.g. incorporation of fluorescent dyes such as FURA-2 that are sensitive to calcium (FLIPR calcium assay kit (Granas et al. 2005)) or a luminescent response from photoproteins such as apoaequorin when they are exposed to calcium (Stables et al. 1997). More recently, Vilardaga et al. described the use of FRET to monitor the beginning of the signalling cascade, rather than the intracellular response of the messengers within the cell. Vilardaga’s group monitored changes in G-protein coupled receptor conformation post activation in a cell-based model (Vilardaga et al. 2003). Janetopoulos et al, Yi et
al. and Bunemann et al. have similarly used the FRET system to monitor changes in heterotrimeric G-protein arrangement upon GPCR activation \textit{in vivo} (Bunemann, Frank & Lohse 2003, Janetopoulos, Jin & Devreotes 2001, Yi, Kitano & Simon 2003). However some of these procedures are technically difficult, may involve multiple assay steps, and require the maintenance of cell cultures. The use of the whole cell in biosensor devices may not prove useful in field applications or within an environment which is detrimental to the cell.

\subsection*{1.5.2 Cell-free assays}

As an alternative to cell-based assays, GPCR activation has been monitored \textit{in vitro} by the use of both radioactive and fluorescent methods.

\subsubsection*{1.5.2.1 Ligand binding}

Traditional pharmacological studies of GPCRs have commonly used radioactive forms of known GPCR antagonists or inverse agonists. Radioligand binding methods are widely used due to the relative ease with which they can be done and the availability of radioligands (Bylund & Toews 1993). Radioligand experiments are generally filtration-based methods which allow for the capture of protein-bound radioligands and removal of non-bound radioligands. Most analysis of radioligand binding experiments are based on the law of mass action, which includes the assumptions that a) binding occurs between the ligand and receptor due to diffusion and is dependent on the correct molecular structure of the ligand as to whether it binds to the receptor; b) the ligand and receptor remain bound for a period of time dependent on the affinity of the receptor for the ligand; c) once dissociated, the ligand and receptor are the same as before they bound; and d) equilibrium of the interaction is established when the ligand/receptor complex forms at the same rate as they dissociate.

Fluorescent ligands provide an alternative to the use of radioactive isotopes in GPCR ligand binding studies. However, non-peptide fluorescent ligands are reportedly more difficult to produce with affinity and efficacy unaltered from that of the original ligand (Middleton & Kellam 2005). Consequently, many more radioligands than fluorescent GPCR ligands are commercially available.
One limitation in the use of ligand binding assays to monitor GPCRs is that, alone, they do not provide information about the ligand efficacy. Additionally, they commonly require a separation step to remove unbound ligand from bound ligand, decreasing the likelihood that this format would be applicable to high-throughput purposes.

1.5.2.2 G-protein activation

Guanine nucleotide exchange provides a functional assay system of a very early event in the GPCR-mediated signal transduction process. GTP analogues have been reported to monitor both the GTPase activity ($^{32}$P]GTP release) (Windh & Manning 2002) and the receptor-induced activation of the G$\alpha$ subunit ($^{35}$S]GTP$\gamma$S binding assay) (Harrison & Traynor 2003). The $^{35}$S]-GTP$\gamma$S binding assay measures the level of G-protein activation following agonist activation of a GPCR (by its cognate ligand), by determining the binding of the non-hydrolysable analogue $^{35}$S]-GTP$\gamma$S to G$\alpha$ subunits (Windh & Manning 2002, Harrison & Traynor 2003, Milligan 2003). Since the $\gamma$-thiophosphate bond is resistant to hydrolysis by the GTPase associated with the G$\alpha$ subunit, G-proteins are prevented from reforming as a heterotrimer and thus $^{35}$S]-GTP$\gamma$S labelled G$\alpha$ subunits accumulate and can be measured by filtration assays, counting the amount of $^{35}$S]-label captured. Recent studies utilizing $^{35}$S]-GTP$\gamma$S together with use of antibody coated scintillation proximity assay (SPA) beads provides a homogenous assay format for the detection of receptor induced activation of the G-proteins (DeLapp 2004). Following receptor activated $^{35}$S]-GTP$\gamma$S exchange for GDP on the G$\alpha$ subunit, the G-protein complex is captured by the anti-IgG coated SPA beads. $^{35}$S] bound in close proximity to the beads excites the scintillant inside the beads to give off a light signal that is read by a microplate scintillation counter.

While the scintillation proximity assay (Ferrer et al. 2003) is a method by which the $^{35}$S]GTP$\gamma$S binding assay becomes a homogeneous assay, it still requires the use of radioactive compounds. There are several disadvantages in using radiolabels for the assays of the type described above, including high costs, radioactive waste, health hazards, and the need for special equipment and licenses. For this reason fluorescent labels for use in G-protein assays have been developed. Fluorescent assays are a popular choice as they are useful for studying processes in real time. Fluorescent nucleotide analogues of GTP have been developed as probes for G$\alpha$ subunit activation.
N-methylanthraniloyl (MANT) conjugates of GTP have been used to study the concentration and kinetics of Gαi/o subunits (Remmers & Neubig 1996, Remmers, Posner & Neubig 1994). The MANT-GTP conjugates were reported to show G-protein binding by the relatively large fluorescence increase, observed due to the resonance energy transfer from tryptophan amino acids in the Gα subunit to the MANT-labelled guanine nucleotide. However, MANT-GTP did not bind equally well to all types of Gα subunits. A more useful label was reported with the development of BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-alkyl) conjugated guanine nucleotides (Kimple et al. 2003). However, very high concentrations of BODIPY-FL-GTPγS as well as Gα-proteins were required to obtain adequate signals as demonstrated in studies involving G-proteins and their interactions with a regulator of G-protein signalling (RGS) (Kimple et al. 2003). This requirement of high concentrations of G-proteins, along with the need for highly purified components, indicates that these GTP analogues would probably not prove useful in a reconstituted membrane system for measuring signalling activity. To date they have not been used with GPCRs to measure ligand-induced activation of a GPCR but have been limited to reporting only interactions between G-protein subunits.

More recently, there have been studies reporting the use of a non-hydrolysable GTP analogue coupled to the lanthanide, Europium (Frang et al. 2003). This assay utilizes the unique long-lifetime fluorescent properties of the lanthanides (discussed in more detail in section 3.1.4.1) to enable time-resolved fluorescence measurements to be obtained. Time-resolved measurements served to decrease background emission and thus enable the signal measurement in the presence of a receptor membrane preparation. The model system used was the α2-adrenergic receptor, and receptor activation was promoted with the natural agonist, adrenaline. This methodology provides a promising new development in the fluorescence measurements, however, filtration steps were still required in the assay (i.e. it is not a homogeneous assay). Minimal steps in assay procedure would be highly desirable to increase speed and efficiency for high-throughput screening.

This study focuses on the ability to determine G-protein activation in a homogeneous cell-free system with the future implication being the development of assay
miniaturization. The reasoning for this includes the possibility of configuring the GPCR system in a microarray or biosensor format. Miniaturization of cell based assays are limited to the dimensions of the cell (typical mammalian cell \(\sim 10\mu m\)) compromising the ability for high-density, microarray screening formats. A system comprised solely of the G-protein/GPCR complex would allow for adaptation to a relatively high throughput screening platform, the size of which is reduced \(\sim 1000\) fold in comparison to the dimensions of a cell (in a cell-based assay system). As well as the inherent capacity to miniaturise the assay, a cell free system could also allow for a variety of GPCR/G-protein combinations to be patterned onto a sensor surface. For example, Fang et al. have achieved this with GPCR expressing membranes (Fang, Frutos & Lahiri 2002a, Fang et al. 2006). The controlled construction of the cell-free system means there is little interference in the assay system from other intracellular processes or proteins, which is another limitation of cell based assays. Finally, cell free systems may be made robust and have longer term stability and portability than cell based systems as conditions to maintain cell viability are not required (reviewed in (Leifert et al. 2005a)).

1.5.3 Conformational changes in the receptor and/or G-proteins

In addition to ligand binding and GTP binding techniques, receptor activation has also been demonstrated by monitoring changes in conformation of the GPCR or the G-proteins. Most commonly, FRET is used for its ability to monitor changes in the proximity of two fluorophore conjugates (for more information on FRET, see section 3.1.3). Studies involving the changes in intramolecular FRET, between fluorescent protein fusions in both the parathyroid hormone receptor and the \(\alpha_{2A}\)-adrenergic receptor were reported by Vilardaga et al. (Vilardaga et al. 2003). FRET changes have been monitored between a fluorescently tagged \(\beta_2\)-adrenergic receptor and it’s associated G-protein by Galès et al. (Gales et al. 2005), and interactions between G-proteins have been demonstrated by a number of researchers (Janetopoulos, Jin & Devreotes 2001, Yi, Kitano & Simon 2003, Azpiazu & Gautam 2004, Janetopoulos & Devreotes 2002). All of the studies mentioned above demonstrate the adaptability of the FRET technique to monitor signalling of GPCRs in real time within the cell. In this thesis a similar FRET technique is used within isolated lipid extracts from the cell containing the GPCR and G-protein signalling components. In this way, the approach
may enable the production of a high-density array of various GPCRs capable of demonstrating a functional signal response through the action of their associated G-proteins.

1.6 Creating protein arrays

Protein arrays (also referred to as biochips) are commonly composed of miniaturized spots of protein on a solid substrate that permits many tests to be performed at the same time, achieving high-throughput screening. DNA microarrays have been used extensively to monitor expression levels of mRNA encoding for specific genes within an organism, but mRNA level and protein level do not always correlate (Heller 2002). Analytical protein arrays are designed to provide an alternative measure of the relative abundance of proteins in a given sample (Hall, Ptacek & Snyder 2007). Whereas functional protein arrays, (investigated within this thesis) are concerned with protein-protein, protein-peptide, protein-DNA, and protein-small molecule interactions (Hall, Ptacek & Snyder 2007).

Protein immobilization is the key to success of microarray technology. It is a challenging task and requires the full retention of protein conformation and activity as well as minimal non-specific protein adsorption to improve detection performance. A variety of surface chemistries for protein immobilization are described in a recent review by Rusmini et al. (Rusmini, Zhong & Feijen 2007), with some of the immobilization strategies including: physical adsorption of the protein onto a surface, covalent immobilization through functional groups of exposed amino acids, and bioaffinity methods including biotin-avidin; hexahistidine tag-Ni$^{2+}$ affinity; and more recently, complementary oligonucleotides (Tang et al. 2008). Arrays of biomolecules can be fabricated using contact or non-contact printing methods (Barbulovic-Nad et al. 2006), both of which are demonstrated in this thesis.

1.6.1 Array reading

Detection of surface bound fluorophores on microarrays is most commonly achieved using microarray readers (a detailed list of microarray readers can be found in a review by Bally et al. (Bally et al. 2006) ), such as the arrayWoRx Biochip Reader (Applied Precision) used in this thesis. Readers can be fitted with lasers or optical filters of
excitation and photomultiplier tubes (PMT) or charge coupled device (CCD) cameras for detection. Alternative methods for label detection include total internal reflection techniques (Taitt, Anderson & Ligler 2005), fiber optic sensor arrays (Walt 2000), surface plasmon resonance enhanced fluorescence (Liebermann & Knoll 2000) and integrated electro-optic systems (Tuan 1998). Additionally, readers are being developed which are capable of time-gated or time-resolved fluorescence spectroscopy (Patounakis, Shepard & Levicky 2006), a feature which is relevant to fluorescent signals arising from TR-FRET discussed within this thesis (chapter 4).

1.7 Membrane protein immobilisation strategies

While the abovementioned protein arrays have their challenges in production due to factors such as orientation and denaturation, this task becomes more complex when the protein naturally resides within the lipid membrane, such as GPCRs. There is substantial interest in reproducing the plasma membrane in a manner capable of housing membrane proteins in a functional environment outside of the intact cell. The ability of lipid biomolecules to self-assemble, provides a means to produce a system which can organise itself (and associated proteins) in their natural form, the lipid bilayer. There are a number of attempts made to find a generic way of integrating the bilayer environment, such as that of a GPCR within a biosensor or biochip platform. Reimhult and Kumar have recently reviewed some of the membrane platforms for sensor substrates (Reimhult & Kumar 2008). These include solid-supported lipid bilayer, tethered lipid bilayers, and black lipid membranes (or free-spanning membranes).

1.7.1 Supported and tethered lipid bilayers

Strategies which may provide a hydrophobic environment for the surface capture of membrane proteins have involved the adsorption of a phospholipid bilayer directly onto a substrate surface. However, the use of supported lipid bilayers attached directly to a solid support has been known to result in the loss of membrane protein mobility and function. One approach to this problem involved separating the supported lipid bilayer by the use of a thin, water swollen polymeric layer (Tanaka & Sackmann 2005). Tethering can also occur via a tethered lipid molecule (Cornell et al. 1997) or the membrane protein itself (Giess et al. 2004). These systems describe a method by which
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to capture pure proteins reconstituted into a pure lipid environment. This thesis investigates the viability of utilizing the lipid extracts of cells over-expressing the membrane proteins of interest, the GPCRs.

1.7.2 Supported lipid bilayers from native membrane sources

The following section describes examples of cell extracts as surface bound sheets. The surface display of membrane extract sheets has been the focus of the group led by Horst Vogel (Danelon et al. 2006, Perez et al. 2006a, Perez et al. 2006b). The membrane sheets are achieved by the tearing of whole adherent cells by the covering of the cell layer with a second adherent poly-L-lysine coverslip and subsequently removing this, along with the torn membrane sheet. An alternative method to achieve a membrane sheet of this nature was produced in the laboratories of William James Nelson by means of sonication (Yamada et al. 2005). A monolayer of mammalian epithelial cells were “de-roofed” by sonication, to provide cell extracts free of the dorsal plasma membrane, nucleus and almost all intracellular membrane organelles.

Patterning of native cell extracts on solid substrates has been achieved with both human erythrocyte membrane extracts and sarcoplasmic reticulum vesicles (microsomes extracted from rabbit muscles). Selective membrane capture onto cellulose templates was accomplished by two methods: 1) Deep UV photolithography to micropattern the cellulose, and 2) Using a PDMS stamp to create protein barriers on a ultra-thin cellulose layer (Tanaka et al. 2004). Both of these techniques were able to immobilize the Anion exchanger 1 (also known as band 3) membrane protein within the plasma membrane of human erythrocyte (without solubilisation or purification of the transporter protein) (Tanaka et al. 2004). Patterns of membrane protein containing cell extracts have also resulted from direct printing onto array platforms. Reports by Hong et al. and Fang et al. have both provided evidence of functional GPCRs printed onto solid supports (Fang, Frutos & Lahiri 2002a, Hong et al. 2006, Fang, Frutos & Lahiri 2002b).

Micropatterning of these native membrane sheets or reconstituted membrane protein systems by any of the abovementioned methods makes sub-compartments for various reactions or binding events accessible for monitoring. Partitioning of membranes allows for individual analytes or interacting components to be investigated, as well as
providing the potential for parallel experiments within an array or biosensor platform.

1.7.3 Immobilised vesicles

In addition to tethering planar lipid bilayer supports on solid substrates, incorporating isolated reactor vessels or protein carriers in the form of lipid vesicles has also been investigated. Again, the tethered supports maintain the fluidity of the membrane environment, whilst retaining an adequate distance from potential interferences from the substrate. In this case, however, instead of providing a single bilayer system which would require adequate surface patterning for partitioning of reaction systems, the individual vesicles provide these isolated compartments. These vesicles can provide an aqueous interior for soluble protein immobilisation (e.g. providing nanoreactors for enzymes), in addition to providing a fluid lipid bilayer for the incorporation of membrane proteins (as reviewed recently by Christensen and Stamou (Christensen & Stamou 2007)). Boukobza et al. reported the assembly of surface attached phospholipid vesicles via a biotin- streptavidin interaction (Boukobza, Sonnenfeld & Haran 2001). An alternative tethering approach was provided by Niemeyer and coworkers (Niemeyer et al. 1994) in the form of complementary oligonucleotides, a technique investigated for its ability to capture vesicles site-specifically onto a surface and further discussed in section 4.1.3 of this thesis.

1.8 Optical and biophysical techniques to monitor GPCR function

Surface analytical tools offer the possibility for extensive and detailed investigations of biomolecules and biomolecular interactions. These interactions include those that take place on or within a lipid environment. Some of the biophysical techniques that could be used to monitor such interactions include atomic force microscopy (AFM) (Richter & Brisson 2003), surface plasmon resonance (SPR) (Karlsson & Stefan 2002, Rao et al. 2002, Bieri et al. 1999), and quartz crystal microbalance with dissipation (QCM-D) used for piezoelectric crystal sensing (further discussed in chapter 4) (Hook et al. 2001, Wu 1999). Techniques such as these are effective at showing mass changes (e.g. loss of G-protein subunit upon receptor activation), but have limitations if the G-protein cannot be lost to solution (e.g. trapped beneath a lipid bilayer or within a vesicle). In this thesis, the strategy of using optical techniques to measure GPCR activity has been employed as
it has the potential to show changes in spatial protein orientation regardless of how the proteins are captured on a surface. This is especially important using the native vesicles which are described in this thesis as the orientation of the receptors and associated G-proteins is unknown. Investigations into GPCR function using optical techniques have been demonstrated using fluorescence polarization (FP) (Jones et al. 2008), flow cytometry (Waller et al. 2003, Sklar et al. 2002, Simons et al. 2003), total internal reflection fluorescence (TIRF) (Martinez et al. 2003), microarray scanning (Fang, Frutos & Lahiri 2002a, Fang et al. 2006, Fang, Frutos & Lahiri 2002b, Fang et al. 2002), and plasmon waveguide resonance (PWR) (Alves et al. 2003, Hruby & Tollin 2007, Devanathan et al. 2004, Alves et al. 2004); (all of which are described in more detail by McMurchie et al. (McMurchie & Leifert 2006)).

This thesis focuses on the use of fluorescent techniques to monitor GPCR activation whilst such proteins are captured on a platform suitable for a sensing device or a high-density array chip. FP techniques are commonly used to monitor the binding event of a small fluorescent ligand to the target receptor, but such technologies are not suitable when the fluorescent molecule is of similar size to its binding partner (as in the case of the G-proteins). Flow cytometry is generally used for cell-based or bead-based measurements. Here, results were obtained using confocal microscopy and microarray scanners (see section 1.6.1) to monitor fluorescent ligand binding onto GPCR arrayed slides as well as binding of fluorescently labelled G-proteins to the GPCR containing vesicles. In addition to this, a scanner based on evanescent field fluorescence, known as a zeptoREADER microarray imaging system (Zeptosens, a division of Bayer Schweiz, AG, Switzerland) was used by a collaborator, Marta Bally, to increase the signal to noise when measuring bound fluorescent ligand. Results are further described in a collaborative publication (Bailey et al. 2009) (Appendix 6.10). Advantages of evanescent wave based techniques can be attributed to surface specific excitation which aids in reducing background noise. This increases the sensitivity of the technique; indeed the zeptoREADER reports detection limits in the zeptomolar ($10^{-21}$ M) range.

1.9 Chapter summary

In this thesis GPCRs are investigated for their application as a biorecognition molecule in biosensor and biochip devices primarily due to their large range of ligands (e.g
olfactory GPCRs are specific for certain volatiles, and biosensors of volatiles could have a multitude of potential applications, particularly for sensing hidden entities such as explosives or for monitoring food quality), and their importance as therapeutic targets (e.g. high-throughput drug screening technology is highly desirable).

Current cell-based GPCR assays are limited in their miniaturizability, stability and portability (due to the need to maintain cell vitality). Cell-free methods most commonly require filtration steps within the assay design to remove unbound nucleotides or ligands, whereas fluorescence techniques such as TR-FRET create a homogeneous assay. In this thesis, the TR-FRET technique is used for assays with isolated membrane extracts from the cell containing the GPCR and G-protein signalling components. The approach aims to demonstrate a strategy whereby an array of various GPCRs are able to exhibit a functional signal response through the action of their associated G-proteins. Reducing steps within the assay design will aid in creating a high-throughput format for screening purposes. The assay design also has the potential to monitor allosteric modulators of GPCR activity as the functional activity of the receptor is measured by the reaction of the G-protein molecules, not simply the ligand binding event itself. Additionally, the G-proteins themselves can be monitored as potential therapeutic targets.

The use of native vesicles as immobilised carriers of the receptors on the sensor or chip surface aims to provide maximum freedom for the receptor to undergo conformational changes and interactions with associated proteins, while providing the closest possible environment to that of the intact cell in order to minimise receptor denaturation.

1.10 Scope of thesis

This thesis is composed of three results chapters. Each chapter begins with an introduction specifically for results within that chapter. The chapters cover protein production, assay development and protein surface capture.

- In order to produce functional GPCRs for assay and array development chapter two covers the expression and preparation or purification of GPCRs and G-proteins used throughout the thesis. The expression and functionality of the
proteins are addressed using established radioligand and radionucleotide binding assays.

- In order to develop a homogeneous assay system with the potential to be used as a transduction system for high-throughput screening in solution and/or on a biosensor or biochip platform, **chapter three** covers the characterization and utilization of a time-resolved fluorescence resonance energy transfer assay system.

- In order to demonstrate whether the receptors could be captured onto a surface and remain functional for biosensor or biochip applications, **chapter four** then shows GPCR immobilisation using the high affinity interactions between two complementary single stranded oligonucleotides. This chapter covers the immobilisation of native membrane vesicles containing GPCRs. The chapter then introduces a potentially novel system for membrane protein array production which utilizes an alternative form of lipid environment to the vesicle or liposome, known as the cubosome.

- The final chapter of the thesis will summarise the general outcomes of the thesis and address potential future directions for this research.
2 GPCR and G-protein expression, preparation and purification

2.1 Introduction

This chapter discusses the expression and preparation or purification of the GPCR and G-protein constructs used throughout this thesis. GPCR’s are notoriously difficult to purify in their active form and throughout most of the experiments in the following chapters, the GPCRs were maintained in their crude cell membrane environment in an enriched extract. The $G_\alpha$ and $G_\beta\gamma$ protein subunits were expressed in $Sf9$ cells and purified for use in functional reconstitution experiments. These were carried out to determine whether the GPCRs maintained the ability to functionally couple to their messenger proteins. The GPCR subtypes used throughout this thesis are introduced and their physiological and pathophysiological roles within the body explored. The physiological functions of the model receptors used throughout this study, provides important insight into the range of roles the receptors play within the body and the significance of their place in the drug discovery and diagnostic fields. This establishes the applications that lie with developing the assay and array techniques addressed in following chapters.

2.1.1 Choosing an appropriate expression system

2.1.1.1 GPCRs

Cloned GPCRs have previously been expressed in a variety of expression systems including; $E. coli$, yeast, insect cells, and mammalian cells (for a summary of the advantages and disadvantages for these systems please refer to Table 2.1.1-1). The main concerns associated with the $E. coli$ expression system arise because the native system is incapable of carrying out the post-translational modifications required for most receptor types (as reviewed in Escribá et al. (Escribá et al. 2007)). The lipid composition and the reductive environment of the $E. coli$ cytoplasm may also hinder the correct binding properties and folding of the receptors, respectively (as reviewed in Sarramegna et al. (Sarramegna et al. 2003)). The yields of GPCRs expressed in the $E. coli$ system are also
hampered by the toxic effects when the receptor inserts into the bacterial membrane. Grisshammer and colleagues have conducted studies into integrating functional GPCRs into the E.coli inner membrane and successfully expressed and purified the Neurotensin receptor with fusion proteins such as the maltose binding protein (Grisshammer, Duckworth & Henderson 1993), or thioredoxin (Tucker & Grisshammer 1996) which helped increase receptor expression levels. Additionally, they have produced this receptor with the tobacco etch virus protease recognition sites at appropriate locations on the receptor so as to enable the isolation of the receptor from its fusion proteins (White et al. 2004).

Unlike E.coli, yeast cells possess endogenous GPCRs and G-proteins and are capable of performing the required post-translational modifications for functional GPCR production (Reilander & Weiss 1998). There are a number of yeast strains that have been used for the production of GPCRs: S. cerevisiae (Payette et al. 1990), Sz. Pombe (Sander et al. 1994), and P. pastoris (Weiss et al. 1995). One of the disadvantages of the yeast system lies in the differences in lipid composition of the membrane, which has been shown to alter ligand binding properties of the mammalian μ-opioid receptor expressed in S. cerevisiae (Lagane et al. 2000). Additionally, the yeast cells have a cell wall that is generally more difficult to lyse than other cell types, which potentially may dictate increased difficulties in the preparation of functional membrane extracts.

The baculovirus/insect cell system performs the relevant post-translational modifications (with the exception of some glycosylation processing (Kost, Condreay & Jarvis 2005), a process which may be linked to cell surface expression levels of the GPCRs rather than the function of the receptor (Tansky, Pothoulakis & Leeman 2007)). Additionally, this expression system has the added advantage of increased GPCR expression yields as compared to the yeast system. The expression of the foreign gene encoded for in the baculovirus construct (in this case the GPCR of interest), is driven by a strong promoter, the polyhedron promoter, which is highly transcribed in the late stages of infection (Luckow et al. 1993); (for more details refer to section 2.1.2).

The mammalian expression system is not only capable of providing correct folding and modifications to the protein, but also able to provide the environment most acceptable
for a mammalian protein. One advantage of the insect cell system over the mammalian cell expression system is that lower levels of endogenous GPCRs are expressed in the Sf9 insect cells compared with mammalian cell expression systems. Additionally, the insect cell system is a more viable option (in terms of cost) for scaling up the production of the protein of interest. Using the insect cell expression system also avoids some of the health and safety issues associated with working with mammalian cells.

### 2.1.1.2 G-proteins

Similar to the GPCRs mentioned in the previous section, recombinant G-proteins can differ when produced in different expression systems. Wall et al. (1995) elucidated the crystallographic structure of the heterotrimer using recombinant rat Gα subunits expressed in E.coli (Wall et al. 1995). Some studies have used these recombinant bacterial proteins to monitor GDP-GTP exchange on the Gα subunit (Remmers et al. 1999). To perform studies with Gα structures more closely related to human G-proteins, the addition of a myristoyl group by co-transfection of E.coli with the N-myristoyl transferase vector pBB131 has been commonly adopted (Remmers & Neubig 1996, Sarvazyan, Remmers & Neubig 1998), and further post-translational modifications such as palmitoylation have been carried out on the Gα subunit in vitro (Cao & Huang 2005). It is commonly believed that these post-translational modifications of the Gα subunit along with the prenylation of γ-subunits provides a hydrophobic domain or anchor, potentially for interaction with the lipid bilayer and perhaps hydrophobic domains of other proteins (Morris & Malbon 1999). While these recombinant bacterial Gα proteins have been reported to exchange and hydrolyse guanine nucleotides, and interact with βγ subunits, results indicate much lower affinities compared to Sf9 derived or mammalian Gα subunits (Linder et al. 1990) possibly due to the lack of post-translational lipid modifications of the subunits. Again, both yeast and mammalian systems can be used for G-protein production and both systems are capable of maintaining the incorporation of the aforementioned lipid additions. However, in this study, baculovirus infection of Sf9 insect cells has been chosen as the expression system for G-protein production as has been previously published in the literature (Graber, Figler & Garrison 1992).
Table 2.1.1-1 Comparison of the main advantages and disadvantages of various commonly used expression systems to obtain GPCRs and/or G-proteins (modified from (McMurchie & Leifert 2006))

<table>
<thead>
<tr>
<th>Expression System</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria e.g., Eschericia coli spp.</td>
<td>• Many host species to choose from&lt;br&gt;• Many DNA expression vectors available&lt;br&gt;• Relatively cheap&lt;br&gt;• Fast process and easy to scale-up&lt;br&gt;• Yield can be very high</td>
<td>• Prokaryotic, not eukaryotic&lt;br&gt;• Truncated proteins can be produced&lt;br&gt;• The expressed proteins often do not fold properly and so are biologically inactive&lt;br&gt;• Insufficient post-translational modifications made e.g., GPCR glycosylation, G-protein palmitoylation&lt;br&gt;• Overexpression can be toxic to the host cells</td>
</tr>
<tr>
<td>Yeast e.g., Saccharomyces cerevisiae</td>
<td>• Eukaryotic&lt;br&gt;• Fast process and relatively easy to scale-up&lt;br&gt;• Yield can be very high&lt;br&gt;• Relatively cheap&lt;br&gt;• Performs many of the post-translational modifications made to human proteins</td>
<td>• Cell wall may hinder recovery of expressed proteins&lt;br&gt;• Presence of active proteases that degrade foreign (expressed) proteins, therefore may reduce yield</td>
</tr>
<tr>
<td>Insect e.g., Spodoptera frugiperda Sf9, Hi-5</td>
<td>• High levels of expression&lt;br&gt;• Correct folding&lt;br&gt;• Post-translational modifications similar to those in mammalian cells</td>
<td>• Expensive to up-scale&lt;br&gt;• Slow generation time&lt;br&gt;• Difficult to work with&lt;br&gt;• Glycosylation limitations</td>
</tr>
<tr>
<td>Mammalian e.g, CHO, HEK, COS</td>
<td>• Good levels of expression&lt;br&gt;• Correct folding and post-translational modifications</td>
<td>• Relatively low yields&lt;br&gt;• Very expensive to up-scale&lt;br&gt;• Slow generation time&lt;br&gt;• Difficult to work with&lt;br&gt;• Health and safety implications involved</td>
</tr>
</tbody>
</table>

2.1.2 Baculovirus amplification and infection

The baculovirus expression system is the most widely used for the production of recombinant proteins in insect cells. The system works by the introduction of the foreign gene of interest into a non-essential region of the viral genome via recombination with a transfer vector containing the cloned protein of interest. Expression of the foreign gene is driven by the polyhedron promoter of the Autographa californica nuclear polyhedrosis virus (AcNPV) which is highly transcribed in the late stages of infection. The commercially available Bac-to-Bac® system (Invitrogen) has been developed, and is commonly used by researchers to generate recombinant baculovirus constructs within a 7-10 day time period. The ease and speed of this system is primarily due to the use of the *in vivo* bacterial transposition method developed by Luckow et al. in 1993 (Luckow *et al.* 1993), which involves the site-specific transposition of the foreign gene from a donor plasmid to a cloned baculoviral DNA (or
‘bacmid’). This bacmid can be used to transfect insect cells and begin amplification of the virus. Amplification and infection of the Sf9 cells with baculovirus stock is carried out with a multiplicity of infection (MOI) of 0.001-0.1 and 1-2 respectively, according to the following formula (as described in manufacturer’s instructions [Invitrogen]):

\[
\text{Innoculum required (ml)} = \frac{\text{Desired MOI (PFU/cell)} \times (\text{Total number of cells})}{\text{Titer of viral innoculum (PFU/ml)}}
\]

Functional GPCRs and G-proteins have been successfully expressed using the baculovirus system (as reviewed in the following papers (Bouvier et al. 1998, Massotte 2003)).

2.1.3 Model receptors

This section introduces the receptor types used throughout this thesis. It is important to understand the functional roles of the receptors to realise their significance in the fields of drug discovery and diagnostics.

2.1.3.1 \( \alpha_{2a} \)-adrenergic receptor

The \( \alpha_{2a} \)-Adrenergic receptor (\( \alpha_{2a} \)-AR) is a subtype of the adrenergic receptor (or adrenoreceptor) family. The adrenergic receptor family is composed of five members, the \( \alpha_1 \) and \( \alpha_2 \) groups as well as the \( \beta_1 \), \( \beta_2 \) and \( \beta_3 \) groups. Three human \( \alpha_2 \)-adrenergic receptor subtypes exist, these are the \( \alpha_{2a} \), \( \alpha_{2b} \) & \( \alpha_{2c} \). The porcine \( \alpha_2 \)-adrenergic subtypes are pharmacologically more related to the human subtypes compared with the \( \alpha_2 \)-adrenergic receptors of other species (Wikberg-Matsson et al. 1995), and it is this sequence that was available and therefore used in these studies. The \( \alpha_2 \)-adrenergic receptor subtypes have a large third intracellular loop, the region of the receptor that has been implicated in the coupling of G-proteins, as seen by the alteration of this region in signalling assays (Jewell-Motz et al. 1998). The \( \alpha_{2a} \)-adrenergic receptor transduction pathways include stimulatory and inhibitory effects on adenylyl cyclase activity; inhibition of Ca\(^{2+} \) channels; and opening of K\(^+ \) channels (Wade et al. 1999, Docherty 1998). The receptors predominantly couples to the G\( \alpha_{i/o} \) subunit family and can also signal through the G\( \alpha_6 \) subunit. In vitro assays of purified \( \alpha_{2a} \)-adrenergic receptors, reconstituted into lipid vesicles are capable of signalling through their G-protein
subunits (Kurose et al. 1991). The in vitro reconstitution system has also been shown to work with crude membrane extracts of the cells expressing the α2a-adrenergic receptor (Leifert et al. 2005b).

### 2.1.3.1.1 Physiology

The α2a-adrenergic receptors are found in a wide range of tissues including the central nervous system (CNS), platelets, pancreas and adipose tissue. The action of the receptors e.g. in smooth muscle cells, platelets and leukocytes, is mediated by circulating catecholamines (predominantly adrenalin). Actions of the α2a-adrenergic receptors include hypotensive effects (Altman et al. 1999, Makaritsis et al. 1999), and activity as negative modulators for neurotransmitter release such as noradrenaline which impacts on monoamine metabolism in the brain. The α2a-receptor is also implicated for it’s effects involving sedation, analgesia, and hypothermia as demonstrated in studies involving mouse models (Kable, Murrin & Bylund 2000).

### 2.1.3.1.2 Pharmacology

α2-adrenergic receptor agonists such as clonidine, metetomidine, and brimonidine are used to treat patients with hypertension, glaucoma, tumor pain and postoperative pain or to help minimise the symptoms of overactivity during drug withdrawal (e.g. clonidine and lofexidine for opiate withdrawal symptoms (Gerra et al. 2001) ) (Philipp, Brede & Hein 2002). α2-adrenergic receptor antagonists have been used in therapeutics mainly for the treatment of depression, diabetes, and potentially have therapeutic actions for cardiovascular disease, obesity, Raynaud’s disease (a vascular disorder that effects blood flow to the extremities) and male sexual dysfunction (as reviewed by Gentili et al. (Gentili et al. 2007) ).

Receptor ligands (such as the drugs mentioned above) all possess affinity for the receptor (the property that associates the compound with the receptor), while only some have efficacy to produce a response. Affinity values for agonist and antagonists to the α2a member of the α2-adrenergic receptor family, which have pharmacological importance and relevance for their use within this thesis are shown in table 2.1-2.
Chapter Two

Table 2.1.3-1 Binding affinity (K_i)* (mean values) and antagonists at human cloned α2a-AR in CHO-cell membrane (Gentili et al. 2007)

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* affinity is determined relative to [3H]-RX821002

Clonidine is known as a partial agonist, it does not give a full physiological response as does a full agonist (Gentili et al. 2007).

The α2a-adrenergic receptors have been implicated in a number of disease states as previously mentioned. However, due to the lack of receptor specificity, there are also side effects which limit the use of some of these antagonists. E.g. yohimbine, when intravenously administered in healthy human subjects, produces significant symptoms of increased CNS adrenergic activity such as stress and anxiety (Cameron et al. 2000).

One way to avoid side effects due to lack of specificity includes the development of more specific receptor subtype selective ligands. One of the aims of this thesis is to examine the current, and possible future methods by which testing drug actions on GPCRs can be made easier and quicker, so that compounds can be screened over an array of receptor subtypes and cross-reactivity between each receptor can be monitored and therefore potentially minimised.

2.1.3.2 M2-muscarinic receptor

There are five distinct muscarinic receptor subtypes, these include the M1, M2, M3, M4 and M5 receptors. The human sequence of the M2-muscarinic receptor (M2R) was investigated during the course of this thesis. The 3rd intracellular loop of the M2R is larger than most other receptors within the class A family of GPCRs (with the exception of the α2a-adrenergic receptor as described previously). The carboxy-terminal end of
this intracellular loop region is implicated with M₂R:G-protein coupling (Liu et al. 1996) and the M₂R signals through the G\(\alpha_{i/o}\) signalling pathway to inhibit adenylyl cyclase activity.

Muscarinic receptors have previously been purified from Sf9 cells via a histidine tag (Hayashi & Haga 1996) and c-myc or FLAG tag (Park et al. 2001). The receptors were shown to maintain their G-protein coupling capacity post-purification (Hayashi & Haga 1996). These receptors have also been shown to function within crude membrane preparations composed of lysed Sf9 cells (Slessareva & Graber 2003), as is also shown in this thesis.

2.1.3.2.1 Physiology

One of the main functions of the M₂ and M₃ subtypes involves the regulation of contractility of the detrusor urinae muscle (the muscular coating of the bladder). This muscle is primarily responsible for urinary bladder emptying (Wang, Luthin & Ruggieri 1995). These two subtypes are also predominant in the smooth muscle of the gut (Abrams et al. 2006). M₂Rs are found throughout the brain, and play an important role in cognitive function (Seeger et al. 2004). Acetylcholine is the circulating neurotransmitter primarily responsible for muscarinic function. In a similar manner to the adrenergic receptors mentioned above, the muscarinic receptors are also important for neuronal excitability, but also for the negative feedback regulation of acetylcholine release (Abrams et al. 2006). M₂Rs are also found in the eye, where they may play a role in pupillary constriction and dilation (Matsui et al. 2002, Jumblatt & Hackmiller 1994). M₃Rs are also the predominant form of the muscarinic receptor found in the mammalian heart. Stimulation of the M₂R decreases the rate and force of contractility of the heart. Therefore antagonists of the M₂R act by increasing resting heart rate (for further information there are a number of relevant reviews on this topic (Brodde et al. 2001, Brodde & Michel 1999)).

2.1.3.2.2 Pharmacology

M₂R antagonists such as atropine, and agonists such as acetylcholine, have important roles in the regulation of contractility of the heart. Atropine has clinical uses in cases of bradycardia. Pirenzepine is primarily known for it’s ability to antagonise the M₁ subtype
of the muscarinic receptor family, however, it also displays inverse agonist activity for the \( \text{M}_2 \) subtype (Daeffler et al. 1999). In this thesis, fluorescently labelled pirenzepine (BODIPY-pirenzepine) was used (during later array based experiments; see chapter 4) and therefore its binding characteristics (in the non-fluorescently labelled form) to the \( \text{M}_2 \) subtype were initially investigated in this chapter. The affinity of the muscarinic ligands used in this thesis are shown in table 2.1-3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Agonist ( K_i ) (μM)</th>
<th>Inverse Agonist ( K_i ) (μM)</th>
<th>Antagonist ( K_i ) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol</td>
<td>1.8 ( \mu )M</td>
<td></td>
<td>0.63 nM</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>0.35 ( \mu )M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td></td>
<td>0.63 nM</td>
<td></td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>0.4 ( \mu )M</td>
<td>0.303 ( \mu )M</td>
<td>8.43 (3.7 nM)</td>
</tr>
<tr>
<td>Telenzepine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*affinity is determined relative to \( [3H]-\)Scopolamine

\( \Phi \)Sf9 cells (Moriya et al. 1999)

\( \Psi \)M \( \text{M}_2 \)R in rat heart cells (Jacobson, Karton & Baumgold 1992)

Telenzepine is a \( \text{M}_1 \)R antagonist, but also displays a high affinity for the \( \text{M}_2 \)R (3.7 nM). An analog of telenzepine, known as telenzepine amine congener (TAC) was constructed by the laboratory of Kenneth Jacobson in the early 90’s (Jacobson, Karton & Baumgold 1992, Karton et al. 1992) and was obtained for these studies from the National Institute for Medical Health (NIMH) in the USA. As the name suggests, the TAC compound displays an exposed amino group, which enabled the fluorescent labelling of the ligand via NHS-succinimidyl ester linkage. The fluorescently labelled ligand was used to confirm the presence and function of \( \text{M}_2 \)R in later array based experiments (see appendix 6.7). The binding affinity for the TAC molecule was investigated in section 2.3.2.

### 2.1.3.3 \( \text{H}_1 \)-histamine receptor

There are four histamine receptor subtypes, the \( \text{H}_1 \), \( \text{H}_2 \), \( \text{H}_3 \) and \( \text{H}_4 \). The human \( \text{H}_1 \)R
sequence was first cloned, expressed and characterised in the early 90’s (De Backer et al. 1993), it is this sequence that has been expressed using the baculovirus system in this thesis. The $H_3R$ is generally considered a $G_{\alpha q/11}$ coupled receptor which initiates calcium mobilisation within the cell following receptor activation. The G-protein binding region implicated in $H_3R$ functional studies is within the third intracellular loop.

2.1.3.3.1 Physiology

The $H_3R$ is distributed throughout the body, primarily within smooth muscle, endothelium and central nervous system tissue. Through the action of the circulating hormone, histamine, this receptor helps to regulate smooth muscle contraction in the airways and gut, as well as vasodilation and vascular permeability. The $H_3R$ is known to be responsible for most of the acute inflammatory responses, including allergic rhinitis.

2.1.3.3.2 Pharmacology

Pyrilamine, also known as mepyramine, is most commonly used in a radiolabelled form for pharmacological studies of the $H_3R$. However, along with other antagonists, pyrilamine has been re-classified from an antagonist to the inverse agonist class due to it’s negative efficacy at the constitutively active $H_3$-histamine receptors observed in COS-1 cells (Fitzsimons et al. 2004, Bakker et al. 2000). The binding affinities for this inverse agonist and other relevant ligands are shown in Table 2.1.3-3.

Table 2.1.3-3 Binding affinity ($K_i$)* (mean values) of agonists, inverse agonists and antagonists at human cloned $H_3R$ in Cos-7 cells (De Backer et al. 1993)

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (μM)</th>
<th>$K_i$ (nM)</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrilamine</td>
<td>2.63</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Triprolidine</td>
<td>7.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loratidine</td>
<td></td>
<td>123</td>
<td></td>
</tr>
</tbody>
</table>

* $H_3R$ from human corneal epithelial cells (CEPI-17-CL4) (Sharif et al. 1998)
* affinity is determined relative to $[^3]H$-pyrilamine
Surface expression of histamine receptors has been reported using a fluorescently labelled histamine (BODIPY-histamine) molecule (Gutzmer et al. 2005, Ohtani et al. 2003). Experiments presented in chapter four utilize this fluorescent ligand for receptor immobilisation studies, and therefore its pharmacological characteristics were first investigated using competitive binding studies as shown in this chapter (see section 2.3.2).

### 2.1.4 Membrane preparations of GPCRs

In order to characterise the expression and functionality of the three GPCRs mentioned above in section 2.1.3, a membrane extract was prepared from lysed Sf9 insect cells. This thesis avoids the solubilisation and purification of the receptors in an attempt to simplify preparation and decrease the number of potentially denaturing steps required to prepare a high throughput screening system for these membrane proteins. Additionally, some receptor types differ from others with regard to the appropriate solubilisation and purification protocols which are effective and retain a functional receptor. Using native extracts avoids the optimisation of these conditions for every receptor possibly required on an array platform. For this reason, the receptors were kept within the native environment of the cell membranes, as presented in this thesis. The receptor preparations also undergo a urea treatment step (Lim & Neubig 2001) which serves to denature endogenous G-proteins and remove other membrane associated proteins within the extract. This enriches the GPCR content within the membrane fraction and allows for their reconstitution with purified G-proteins of the subtype and concentration of choice for each experiment.

### 2.2 Materials and methods

#### 2.2.1 Materials

Baculovirus stocks (Gαi1His, Gγ2, Gγ2His and α2A-adrenergic receptor) were obtained from Prof. R. Neubig, University of Michigan, USA, and Gβ4 and Gβ1 baculovirus stock from Prof. J. Garrison, University of Virginia, USA. GPCR baculovirus stock of the M2-muscarinic receptor was obtained from Adrejis Krumins and Prof. Alfred Gilman, University of Texas, USA, and H1-histamine receptor baculovirus was obtained from
Prof. Willem J. DeGripp, University Medical Center, Nijmegen, Netherlands. $^3$H-[$N$-methyl-$^3$H] Scopolamine methyl chloride was purchased from Amersham Pharmacia Biotech (Buckinghamshire, U.K., [now GE Healthcare]) at a specific activity of 84 Ci/mmol. $^3$H-pyrilamine and $^3$H-MK-912 were purchased from Perkin Elmer Life Sciences (Boston, M.A., USA) at a specific activity ranging between 20-30 Ci/mmol (pyrilamine) and 60-87 Ci/mmol (MK-912), depending on batch number. [$^{35}$S]-GTP$_\gamma$S was purchased from Perkin Elmer Life Sciences (Boston, M.A., USA) at a specific activity of 1250 Ci/mmol. All radioactive isotopes were diluted to nanomolar concentrations and stored at -20°C (-80ºC for [$^{35}$S]-GTP$_\gamma$S) in 25-100 µl aliquots. Glass microfiber filters were purchased from Filtech (Adelab, Australia). Non-labelled GPCR ligands were purchased from Sigma-Aldrich, Australia. Telenzepine Amine Congener was obtained from the National Institute for Medical Health (NIMH), USA as part of their chemical synthesis and drug supply program (http://nimh-repository.rti.org/). Sf9 cells were purchased from Invitrogen, Australia. Nickel-NTA beads were purchased from Qiagen (Pty Ltd. Vic, Australia) as a 50% bead slurry in EtOH and washed with relevant buffer prior to use in purification. Precaste SDS-PAGE gels and appropriate loading buffers were purchased from Bio-Rad as was nitrocellulose for western blots. Rabbit anti-G$\alpha$ (internal) antibodies were purchased from Calbiochem; and Alkaline phosphatase conjugated anti-rabbit antibodies were purchased from Sigma-Aldrich. All other chemicals were purchased at highest quality from Sigma Aldrich. Buffers and buffer composition are shown in Table 2.2.1-1.
Table 2.2.1-1 Buffer Compositions

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer</td>
<td>50 mM HEPES pH 8.0, 0.1 mM EDTA, 3 mM MgCl₂, 10 mM β-mercaptoethanol, 0.02 mg/ml phenylmethyl sulfonyl fluoride (PM SF), 0.03 mg/ml benzamidine, 0.025 mg/ml bacitracin and 0.03 mg/ml soybean trypsin inhibitor</td>
</tr>
<tr>
<td>Incubation Buffer</td>
<td>250 mM sucrose, 10 mM Tris, pH 8.0, 3 mM MgCl₂, 0.02 mg/ml PM SF, 0.03mg/ml benzamidine, 0.025mg/ml bacitracin, 0.03 mg/ml soybean trypsin inhibitor</td>
</tr>
<tr>
<td>Wash Buffer</td>
<td>50 mM HEPES pH 8.0, 3 mM MgCl₂, 50 mM NaCl, 10 mM β-mercaptoethanol, 10 μM GDP, 0.02 mg/ml PM SF, 0.03mg/ml benzamidine, 0.025mg/ml bacitracin, 0.03 mg/ml soybean trypsin inhibitor</td>
</tr>
<tr>
<td>Buffer A</td>
<td>20 mM HEPES pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 10 mM β-mercaptoethanol, 0.5% (w/v) polyoxyethylene-10-lauryl ether and 10 μM GDP</td>
</tr>
<tr>
<td>Buffer E</td>
<td>20 mM HEPES pH 8.0, 50 mM NaCl, 10 mM β-mercaptoethanol, 10 μM GDP, 1 % (w/v) cholate, 50 mM MgCl₂, 5 mM imidazole</td>
</tr>
<tr>
<td>Buffer F</td>
<td>20 mM HEPES pH 8.0, 3 mM MgCl₂, 10 mM NaCl, 10 mM β-mercaptoethanol, 1 μM GDP and 0.1% (w/v) cholate</td>
</tr>
<tr>
<td>Transfer buffer</td>
<td>25Mm Tris pH 8.5; 190 mM glycine and 20% methanol</td>
</tr>
<tr>
<td>TBST</td>
<td>150 mM NaCl; 2.7 mM KCl; 25Mm Trizma base, pH 7.4; 0.05% v/v Tween-20</td>
</tr>
<tr>
<td>PBS</td>
<td>137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄</td>
</tr>
<tr>
<td>BSA blocking buffer</td>
<td>1.5% BSA in PBS</td>
</tr>
<tr>
<td>Development buffer</td>
<td>100Mm Tris, pH 9.5; 100Mm NaCl; 50Mm MgCl₂</td>
</tr>
<tr>
<td>TMN</td>
<td>50 mM Tris, 100 mM NaCl, 10 mM MgCl₂; pH 7.6</td>
</tr>
<tr>
<td>TMND</td>
<td>50 mM Tris, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT, pH 7.6</td>
</tr>
<tr>
<td>HEPES preparation buffer</td>
<td>10 mM 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid and 150 mM NaCl</td>
</tr>
</tbody>
</table>

2.2.2 Methods

2.2.2.1 Amplification and infection of baculovirus in Sf9 cells

Sf9 (Spodoptera frugiperda) cells in SF900II media (Invitrogen) were grown in suspension culture at 28°C with shaking (138 rpm in an orbital shaker). Baculovirus stocks were filtered (using 0.2 μm syringe filter or by vacuum filtration using a Millipore Stericup) and added to Sf9 cells at 1-2 x 10⁶ cells/ml to amplify at a MOI of 0.1. Amplified stocks were added to Sf9 cells at 1-2 x 10⁶ cells/ml to infect at a multiplicity of infection of 2 (virus titre was assumed at 5 x 10⁷ PFU/ml unless
otherwise stated). Infected cells were grown for 48-72 hr at 28°C with shaking (138 rpm) prior to harvesting.

### 2.2.2.2 Preparation of membrane extract

Sf9 cell membranes containing either the H₁-histamine receptor or M₂-muscarinic receptor were prepared according to a modified method to remove endogenously expressed G-proteins (Lim & Neubig 2001) via urea treatment, as previously described (Leifert et al. 2005b). The harvested cells were washed in PBS (1000 x g, 10 min with all centrifugation steps carried out at 4°C), then re-suspended in cold lysis buffer (Table 2.2.1-1). Cells were then subjected to N₂ cavitation at 500psi (3400 kPa) for 15 min. Lysed cells were centrifuged (750 x g, 10 min) to pellet unbroken cells and nuclei, then the supernatant was re-centrifuged (100,000 x g, 30 min) to pellet membrane fragments. The membrane fragments were then re-suspended in incubation buffer (Table 2.2.1-1) containing 7 M urea and incubated with mixing for 30 min at 4°C. Urea was diluted to 4 M in the sample preparation prior to pelleting the membrane fragment once again (100,000 x g, 30 min). The fragment was washed twice with incubation buffer prior to re-suspension at a concentration of 1-10 mg/ml. Aliquots of membrane fragments were rapidly frozen (liquid N₂) and stored at -80°C.

### 2.2.2.3 Purification of G-proteins

Frozen membranes (at ≥ 5 mg/ml protein) were prepared as described in section 2.2.2.2 with the exception of the urea treatment step, which is omitted for G-protein membrane preparation. Membranes containing combinations of Gα and Gβγ G-protein subunits were thawed and diluted to 5 mg/ml protein with wash buffer (for buffer compositions refer to Table 2.2.1-1) containing fresh protease inhibitors and 1% (w/v) cholate (final concentration) at 4°C. Membranes were stirred on ice for 1 hr for the extraction step. The sample was then centrifuged at 100,000 x g for 40 min at 4°C and the supernatant collected. The supernatant containing soluble G-proteins (G-protein extract) was diluted 5-fold with ice-cold buffer A (Table 2.2.1-1) prior to column loading. The sample was then loaded onto nickel-nitrilotriacetic acid (Ni-NTA) beads to allow selective binding to hexahistidine-tags. The G-protein extract was added to a 800 μl column and allowed to pass through (under gravity) and the sample collected and re-applied to the column. The column containing hexahistidine-tagged G-proteins was washed with 50 ml buffer
A containing 5 mM imidazole and 300 mM NaCl to remove proteins not specifically bound to the column via Ni\textsuperscript{2+}-histidine interaction. All washing procedures were carried out at 4°C. The non-hexahistidine-tagged G-protein subunits were eluted with 400 μl fractions of buffer E (Table 2.2.1-1) containing freshly prepared 10 mM NaF and 30 μM AlCl\textsubscript{3} (AlF\textsubscript{4}\textsuperscript{-}). The remaining hexahistidine-tagged G-protein subunits were eluted from the nickel column with buffer E containing 150 mM imidazole. Aliquots of elution fractions were then run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for visualisation of the level of purity of the proteins (see section 2.2.2.4). Elution fractions containing the appropriate G-protein subunit were pooled (to ≤ 3.0 ml) and dialysed using a Slide-a-lyzer (Pierce Chemical Company) with 4 x 200ml changes of buffer F, then overnight against 200 ml buffer F (Table 2.2.1-1). Following dialysis, the G-protein subunits were snap frozen in liquid N\textsubscript{2} and stored at -80°C in buffer F. Final protein concentration was determined by Bradford Protein Assay (Bradford 1976). The yields of G-proteins ranged from approximately 0.05-0.5 mg per litre of Sf9 insect cell culture infected with baculovirus at a cellular concentration of 2 x 10\textsuperscript{6} cells/ml. Purity was routinely >95%.

2.2.2.4 SDS-PAGE

Protein samples (20 μl) or Kaleidescope pre-stained standard (Bo-Rad) (8-10 μl) were loaded with an equivalent volume of 2x Laemmli sample buffer (Bio-Rad) containing 715 mM β-mercaptoethanol, and heated at 100°C for 3 minutes prior to loading on a 15% pre-cast gel (Bio-Rad). The polyacrylamide gel was then run at 150 V for 20 minutes, then the voltage was increased to 200 V until the dye front reached the end of the separating gel. The gel was then washed 3 times for 5 min each time in MQ water prior to a 30 min incubation in Coomassie blue protein stain (in 40% methanol/10% acetic acid (v/v)). Gels were destained in 40% methanol/10% acetic acid (v/v) solution for > 2 hours, or until background staining was reduced and bands could be clearly visualised.

2.2.2.5 Western blot

2.2.2.5.1 Transfer

If further protein characterisation and identification was required, western blot analysis
was undertaken. This process begins with a SDS-PAGE gel (without the coomassie staining), run with a pre-stained molecular weight marker (Kaleidescope, Bio-Rad) and relevant protein samples. The gel was rinsed quickly in MQ water after it had been run (as described in section 2.2.2.4), then equilibrated in transfer buffer (Table 2.2.1-1). The gel was then covered with an equivalent size of nitrocellulose membrane (BioRad) and sandwiched between two pieces of filter paper and two sponge pads between the transfer cassette while avoiding any bubbles (all while kept soaked with transfer buffer). Proteins were transferred to nitrocellulose using a 110 V electric field for 1 hour.

2.2.2.5.2 Detection

The nitrocellulose containing transferred proteins were washed for 5 minutes in TBST (Table 2.2.1-1), then blocked with BSA blocking solution (Table 2.2.1-1) overnight. After washing the membrane 3 times in TBST, the primary antibody (rabbit-anti-\(G_\alpha\) [internal]) (Calbiochem) was then added and incubated for 2 hours with gentle rocking. The nitrocellulose membrane was washed again in TBST (3 times) and then incubated with the secondary alkaline phosphatase linked antibody (Sigma-Aldrich) for 1 hour. The membrane was developed after a last wash (3 times) with TBST, by placing the nitrocellulose membrane in development buffer (Table 2.2.1-1) and adding sequentially nitro-blue tetrazolium (NBT) at a concentration of 2.73 mM and then 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) at a concentration of 136 \(\mu\)M for incubation until bands became clearly visible. Development was terminated by washing in MQ water.

2.2.2.6 \(^{3}\)H-Ligand saturation binding curves

To determine receptor-ligand binding specificity, saturation curves were generated using \(^{3}\)H-MK-912 (an adrenaline analogue) for the \(\alpha_{2a}\)-adrenergic receptor, \(^{3}\)H-scopolamine for the \(M_2\)-muscarinic receptor (\(M_2R\)) preparation, and \(^{3}\)H-pyrilamine for the \(H_1\)-histamine receptor (\(H_1R\)) preparation; (for specific activity of radioligands at time of purchase, see section 2.2.1). Reactions were carried out in a volume of 100 \(\mu\)l. Samples were incubated with various concentrations of appropriate tritiated ligand in TMN buffer (Table 2.2.1-1) for 90 minutes with gentle mixing at 27ºC, then filtered over GF/C filters. To determine non-specific binding, an additional concentration curve was generated in the presence of the appropriate receptor antagonists (100 \(\mu\)M atropine,
100 μM triprolidine or 100 μM yohimbine were used as antagonists for the M₂R, H₁R and α₂aAR, respectively, to block the ³H-ligand binding site). Specific binding was calculated by the subtraction of non-specific binding from total binding. Samples were incubated for 90 minutes with gentle mixing at 27°C then filtered over GF/C filters. Filters were washed using 3 x 4 ml washes with TMN buffer. Liquid scintillant was added to the filters and a scintillation counter (Wallac 1410) was used to determine the amount of ³H-ligand bound.

2.2.2.7 ³H-ligand competitive binding curves

To determine the equilibrium dissociation constants for non-radiolabelled ligands, competition curves were generated. In these experiments the binding of a single concentration of radiolabelled ligand was measured in the presence of various concentrations of unlabelled ligand. The radioligand concentrations used were 2 nM for both ³H-pyrilamine and ³H-scopolamine. Compounds that were tested for their relative potencies towards the M₂R included pirenzepine, atropine and telenzepine amine congener (TAC), which were added to the assay at various concentrations between 0-10 μM to compete for ³H-scopolamine binding. Compounds that were tested for their relative potencies toward the H₁R included triprolidine, histamine, and BODIPY histamine, which were added to the assay at various concentrations between 0-10 μM to compete for ³H-pyrilamine binding. Samples were incubated for 90 minutes at 27°C with shaking to achieve equilibrium then filtered over GF/C filters. Filters were washed with 3 x 4 ml of TMN buffer. Liquid scintillation counting was used to determine the amount of ³H-ligand bound. Competition curves provided EC₅₀ values of the unlabelled compounds, which describes the effective molar concentration of the compound that reduces ³H-ligand binding to 50% of the maximum possible response. The EC₅₀ value is directly related to the Kᵢ (equilibrium dissociation constant) for the unlabelled ligand (see formula below). This value is equivalent to the Kᵢ value obtained from competition binding studies.

\[ Kᵢ = \frac{EC₅₀}{1 + \left[\frac{[ligand]}{Kᵢ}\right]} \]
2.2.2.8 $^{35}$S-GTP$_{\gamma}$S reconstitution assay

Functional activity of the receptors was measured by monitoring the activity of the G-proteins. Specifically, receptor induced activation of the G$\alpha$ subunit was demonstrated by a radioactive GTP binding assay as previously described (Leifert et al. 2005b). Purified G$\alpha_{i1}$ or G$\alpha_4$ and G$\beta_4\gamma_2$ or G$\beta_1\gamma_2$ protein subunits at a concentration of 20 nM were introduced into receptor membrane mixtures. Agonist stimulated $[^{35}S]$-GTP$_{\gamma}$S binding studies were performed using a modification of published techniques (Windh & Manning 2002). A reconstitution mix consisting of 0.05 mg/ml M$_2$R, $\alpha_{2A}$-AR or H$_1$R native membrane extracts, 5 $\mu$M GDP, 10 $\mu$M AMP-PNP, 20 nM G-proteins and 0.25 nM $[^{35}S]$-GTP$_{\gamma}$S were prepared in a final volume of 100 $\mu$l in TMND (Table 2.2.1-1) on ice. The reactions were initiated with either buffer (basal) or receptor agonist (1 mM histamine for the H$_1$R and 120 mM carbachol for the M$_2$R or 10 $\mu$M epinephrine for the $\alpha_{2A}$-AR). Specificity of agonist stimulation was determined by introducing a receptor antagonist (100 $\mu$M pyrilamine for the H$_1$R; 100 $\mu$M atropine for the M$_2$R or 100 $\mu$M yohimbine for $\alpha_{2A}$-AR) to block the agonist binding site and hence further signalling through the G-proteins. Samples were incubated for 90 minutes with gentle mixing at 27°C then filtered over GF/C filters. Filters were washed with 3 x 3 ml washes of TMN buffer. Liquid scintillant was added to filters and radioactivity was measured on a beta counter (Wallac 1410) to determine amount of $[^{35}S]$-GTP$_{\gamma}$S bound.

2.2.2.9 Data analysis

Data was analysed using Prism™ (GraphPad Software Inc., San Diego CA, USA). Data shown is mean ± SEM where sample number (n) is greater than or equal to 3, however, if the experiment was conducted in duplicate, data represents mean ± range of duplicates. Where error bars are not visible, they are within the data point symbol (unless otherwise stated). $K_d$ and Bmax values were calculated using non-linear regression analysis for one-site binding. For radioligand competition assays, the effective concentration at 50% maximal response (EC$_{50}$), was calculated using the sigmoidal dose response model of the Prism software.
2.3 Results and discussion

2.3.1 Receptor expression

The radioligand binding assay technique is a powerful method for studying the ligand interactions with receptors. Saturation binding curves were conducted over a 100-fold concentration range of radioligand, this being the recommended guidelines if the system generally conforms to a one-site binding model (Bylund & Toews 1993). Total radioligand binding was conducted in the concentration range of 0-6 nM of radioligand (e.g. $^3$H-pyrilamine in Figure 2.3.1). The non-specific binding of radioligand to the membrane preparation carrying the receptor was investigated by the addition of an excess concentration of 100 $\mu$M of non-radiolabelled ligand (as shown by the linear regression at lower dpm [disintegrations per minute] values in Figure 2.3.1). To determine specific radioligand binding, the non-specific values obtained for each radioligand concentration were subtracted from the total radioligand binding at the same concentration (as shown by the broken line in Figure 2.3.1).

![Graph: 3H-ligand binding to Sf9 membrane extracts containing the H1-histamine receptor.](image)

**Figure 2.3.1** $^3$H-ligand binding to Sf9 membrane extracts containing the H1-histamine receptor: a) (■) Total H1-histamine receptor binding ($^3$H-pyrilamine) in Sf9 membranes 0.5μg protein/100μl assay, 28°C, 90 min in TMN buffer. (▲) Non-specific binding in the presence of 100 $\mu$M triprolidine. (●) Specific binding of $^3$H-pyrilamine to H1-histamine receptors in membrane extract. Representative data, n = 2.

Specific radioligand binding indicates the presence and number of receptors within the Sf9 cell extract preparations. Figure 2.3.1 shows the disintegration per minute (dpm) of
the radiolabel $^{3}$H when examined in a beta counter in the presence of liquid scintillant. This value can be converted to fmoles/mg of total protein in the assay by a conversion factor obtained by determining total counts which are equivalent to the number of moles of $^{3}$H-ligand within the assay volume. The saturation binding curves of all three receptors used throughout this thesis, the $\alpha_{2a}$-adrenergic receptor, the M$_{2}$-muscarinic receptor, and the H$_{1}$-histamine receptor, are shown in Figure 2.3.2.

**Figure 2.3.2 Specific $^{3}$H-ligand binding in Sf9 membranes** a) Specific M$_{2}$-muscarinic receptor bound ($^{3}$H-scopolamine) in Sf9 membranes 1.0µg protein/100µl assay [28°C, 90min in TMN buffer] determined following subtraction of ligand bound in the presence of 100µM atropine. B$_{max}$ = 6.07 ± 0.23 pmoles/mg; K$_{d}$ (apparent) = 0.62 ± 0.08 nM, n = 5. $R^2 = 0.94$. b) Specific H$_{1}$-histamine receptor bound ($^{3}$H-pyrilamine) in Sf9 membranes 1.0 µg protein/100 µl assay [28°C, 90 min in TMN buffer] determined following subtraction of ligand bound in the presence of 100 µM triprolidine, apparent B$_{max}$ = 10.88 pmoles/mg; K$_{d}$ (app) = 1.33 nM , n = 2. $R^2 = 0.98$. c) Specific $\alpha_{2a}$-adrenergic receptor bound binding in Sf9 membranes 0.5ug/100µl assay [28°C, 90 min in TMN buffer] following subtraction of binding in the presence of 100 µM yohimbine. B$_{max}$ = 36.9 ± 3.9 pmoles/mg; K$_{d}$ (app) = 2.2 ± 0.53 nM , n = 3. $R^2 = 0.93$.

The M$_{2}$-muscarinic receptor saturation binding curve resulted in an apparent K$_{d}$ (app) of 0.62 ± 0.08 nM and a B$_{max}$ of 6.07 ± 0.23 pmoles/mg (Figure 2.3.2a). These values are slightly varied from literature values of M$_{2}$R expressed in CHO-K1 cells, in which the saturation binding isotherm using $^{3}$H-scopolamine resulted in a K$_{d}$ = 0.08 nM and a B$_{max}$ of 0.75 ± 0.02 pmoles/mg (Dorje et al. 1991), as well as that of M$_{2}$ receptors.
previously expressed in Sf9 insect cells, of which the saturation binding curve with $^3$H-scopolamine resulted in a $K_d$ value of $0.08 \pm 0.01$ nM (Rinken et al. 1994) or a $K_d$ value of $0.17 \pm 0.03$ nM (Moriya et al. 1999). However the apparent $K_d$ value remains in the low nM range and indicates the presence of the M2R within the membrane. The expression levels of M2R in the Sf9 cells is shown in Figure 2.3.2a ($B_{max} = 6.07 \pm 0.23$ pmoles/mg) and were approximately 10 x higher than in CHO cells which have been noted previously in GPCR expression studies (as reviewed in Sarramegna et al., (Sarramegna et al. 2003)), but similar to expression levels obtained previously by Moriya et al. in Sf9 cells, where a $B_{max}$ value of $6.87 \pm 0.86$ (Moriya et al. 1999) was obtained. The specific $^3$H-pyrilamine binding to the H1-histamine receptor resulted in an approximate $K_d$ (app) of 1.33 nM and a $B_{max}$ of 10.9 pmoles/mg (Figure 2.3.2b). These values agree with literature values quoting the $K_d$ of $^3$H-pyrilamine binding to the human H1-histamine receptor expressed in Cos-7 cells as 1.2 nM, and a $B_{max}$ of 3.4 pmoles/mg (De Backer et al. 1993). Again, the expression levels obtained in the Sf9 cells have exceeded those obtained in the mammalian cell line. $^3$H-MK-912 saturation binding curves with the $\alpha_{2a}$-adrenergic receptor, resulted in a $K_d$ value of $2.2 \pm 0.53$ nM with a $B_{max}$ of 36.9 ± 3.9 pmoles/mg (Figure 2.3.2c) which is a slight variation on the literature value for the cloned guinea-pig (closest sequence similarity to the pig sequence used in this thesis) $\alpha_{2a}$-adrenergic receptor sequence expressed in COS cells, in which the $K_d$ value obtained was $0.81 \pm 0.25$ nM (Svensson et al. 1996). The dissociation constant remains in the low nM range which indicates specific $\alpha_{2a}$-AR binding. In this case, the difference in the $K_d$ values could stem from slight sequence variation between the pig and guinea-pig $\alpha_{2a}$-ARs.

## 2.3.2 Radioligand competition assay

The use of fluorescent ligands for monitoring functional receptor immobilisation in upcoming chapters (chapter 4), required the elucidation of relevant $EC_{50}$ values from various agonists, antagonists and inverse agonists to the M2 and H1 receptors. The fluorescent ligands used in subsequent chapters in this thesis include BODIPY-pirenzepine (Invitrogen, Switzerland) and TAC (telenzepine amine congener)-Alexa; (TAC was provided by the NIMH, Chemical Synthesis and Drug Supply Program and labelled with Alexa 546 NHS [Invitrogen, Australia]). Due to insufficient quantities of
fluorescent labelled ligands being available, the unlabelled pirenzepine and TAC were used in the following competition studies. To give an example of the affinity shift that may occur once a ligand has a fluorescent moiety attached, a preliminary experiment using the BODIPY-histamine ligand was conducted (Figure 2.3.5). Receptor-ligand affinity can be determined using a radioligand competition assay.

![Graph](image)

**Figure 2.3.3 M₂R radioligand competition assay with pirenzepine:** Specific ³H-Scopolamine binding to the M₂-muscarinic receptor was competed for with increasing concentrations of the inverse agonist, pirenzipine. 1 μg membrane protein was incubated with 2 nM ³H-Scopolamine in 100 μl assay volume, in the presence of increasing concentrations (0-10mM) of competing ligand (pirenzepine). The apparent EC₅₀ value of pirenzepine was calculated at 1.14 μM. n = 2, (representative data).

The EC₅₀ value obtained for pirenzepine inhibition of the M₂R was 1.14 μM. This equates to a Kᵢ (equilibrium dissociation constant) of 0.27 μM (calculated using the formula described in section 2.2.2.7). This Kᵢ value is close to the cited 0.303 μM obtained for pirenzepine binding to cloned human M₂R in Sf9 cells (Moriya *et al.* 1999), as shown in Table 2.1.3-2. It is expected that this EC₅₀ value could possibly shift to higher values for the fluorescently labelled pirenzepine, as can be seen with histamine receptor binding parameters in Figure 2.3.5.

Telenzepine has been constructed by researchers in the USA to contain an amine group, giving the analogue telezepine amine congener (TAC) (Jacobson, Karton & Baumgold 1992, Karton *et al.* 1992). The functional group introduced into this compound enables the covalent binding of a fluorescent probe containing a succinimidyl ester. Despite displaying the highest affinity for the M₁-muscarinic receptor subtype, the TAC moiety also binds with high affinity to the M₂-muscarinic receptor subtype (apparent EC₅₀ = 4.6 nM), a concentration very similar to the M₂-specific antagonist, atropine (Figure 2.3.4).
This equates to a $K_i$ value of 1.09 nM, which is within the range of the affinity constant obtained for telenzepine binding to $M_2R$ in rat heart cells ($K_i = 3.7$ nM) (Jacobson, Karton & Baumgold 1992), as shown in Table 2.1.3-2. The telenzepine ligand (TAC) used in the competition assay shown in Figure 2.3.4 displays an additional amine group than the telenzepine ligand referred to in the literature comparison (reporting the $K_i$ of 3.7 nM) (Jacobson, Karton & Baumgold 1992), and this may contribute to changes in their relative affinity. Interestingly, the TAC molecule has displayed a higher affinities for the $M_2R$ than the unfunctionalised telenzepine molecule. This indicates that the fluorescently tagged TAC molecule could be useful in array-based assays introduced in chapter 4 (see appendix 6.7).

![Figure 2.3.4](image)

**Figure 2.3.4 $M_2R$ radioligand competition assay with atropine and TAC**: 1 μg membrane protein was incubated with 2 nM $^3$H-Scopolamine in a 100 μl assay volume, in the presence of increasing concentrations (0-10 μM) of competing ligand. Apparent EC$_{50}$ value for the unlabelled TAC (black ▼) and atropine (red ■) compounds are 4.6 nM and 9.1 nM, respectively; $n = 2$, representative data.

The $H_1$-histamine receptor competition binding curves displayed a high affinity interaction of the receptor with the triprolidine antagonist (apparent EC$_{50} = 153$ nM) (Figure 2.3.5). This equates to a $K_i$ value of 61.1 nM, which is higher than the dissociation constant obtained for triprolidine binding to $H_1R$ in human corneal epithelial cells (Sharif et al. 1998) ($K_i = 7.7$ nM), as shown in Table 2.1.3-3. This change in affinity may be attributed to the different cell types in which the respective receptors are expressed.
Once the histamine agonist is bound to the BODIPY fluorophore, the affinity of the ligand for the H1-histamine receptor decreases by a factor of approximately 35, from a $K_i$ value of 43 $\mu$M (EC50 ~110 $\mu$M) for the non-fluorescent histamine, to a $K_i$ of approximately 1.5mM (estimated EC50 ~ 4 mM) for the BODIPY labelled histamine. The equilibrium dissociation constant for histamine binding to human H1R expressed in COS-7 cells is reported as 24 $\mu$M (De Backer et al. 1993) as shown in Table 2.1.3-3, which is slightly lower than that obtained in Figure 2.3.5.

Changes in ligand binding parameters occur as a result of GPCR:G-protein associations; and this difference may be due to the absence of any G-proteins within the Sf9 membranes used in the competition assays, compared with the H1R’s expressed in the COS-7 cells which are free to associate with endogenous G-proteins. While experiments using the fluorescently labelled histamine were not comprehensive due to a lack of supply of fluorescent ligand, this data indicates a lower affinity of fluorescent ligands to the receptors than its non-fluorescent counterpart. Such a phenomenon that has previously been reported in the literature for the FITC labelled CGP for the β-adrenergic receptor (Heithier et al. 1994). Using fluorescent antagonists to the histamine receptor, such as those synthesized by Li et al. (Li et al. 2003), may be more appropriate due to their increased affinity for the H1R. However, due to commercial availability, BODIPY-histamine was used for these studies.
2.3.3 G-protein purification

The G-protein subunits G\(\alpha_{i1}\), G\(\alpha_{q}\), G\(\alpha_{i1}\)his (the G\(\alpha_{i1}\) subunit containing a hexahistidine tag on the N-terminus), G\(\beta_{4}\) or G\(\beta_{1}\) dimerized with G\(\gamma_{2}\) or G\(\gamma_{2}\)his (the G\(\gamma_{2}\) subunit containing a hexahistidine tag on the N-terminus), were all purified using the procedure described in section 2.2.2.3. Subunits were eluted from the Ni-NTA column either by using AlF\(_4^{-}\) which is capable of activating the G\(\alpha\) subunit as seen previously (Kozasa & Gilman 1995, Hepler et al. 1993) and thereby initiating the separation of the subunits, or by imidazole if the subunit contained a hexahistidine-tag. Six elutions of the void volume of the column were collected and run on SDS-PAGE gels as described in section 2.2.2.3 and 2.2.2.4. SDS-PAGE gels were stained with Coomassie blue protein stain. Expected molecular weights for the proteins as calculated from relevant sequences (G-protein database [http://bioinformatics2.biol.uoa.gr/gpDB/]) were: G\(\alpha_{i1}\) (rat) = 40.3 kDa; G\(\alpha_{q}\) (rat) = 41.5 kDa; G\(\beta_{1}\) (rat) = 37.4 kDa; G\(\beta_{4}\) (mouse) = 37.4 kDa; G\(\gamma_{2}\) = 7.8 kDa. The 6-histidine tag adds a further 840 Dalton onto the molecular weight of the protein. Molecular weight calculations were done from amino acid sequence using protein calculator v3.3 (http://www.scripps.edu/~cdputnam/protcalc.html). However, hexahistidine-tagged G\(\alpha\) was commonly visualised at slightly higher molecular weights, possibly due to changes in migration properties through the gel resulting from the hexahistidine-tag.

![Figure 2.3.6 G\(\alpha_{i1}\)His purification PAGE](image)

**Figure 2.3.6 G\(\alpha_{i1}\)His purification PAGE**: Coomassie Blue stained protein at approximately 45kDa (shown by arrow) indicates the presence of G\(\alpha_{i1}\)his. Marker- Kaleidoscope Prestained standards (Bio-Rad). Lanes from right to left are molecular weight (MW) standard, then following 6 lanes are 150 mM imidazole elutions (1-6) from column.

The concentrations of the G\(\alpha\) of G\(\beta\gamma\) subunits, generally ranged from 1-10 \(\mu\)M or approximately 45 - 450 \(\mu\)g per litre of infected Sf9 culture, note that 1L is approximately equivalent to a total Sf9 membrane protein concentration of 200 mg, which is similar to concentrations reported in the literature, particularly for the G\(\alpha_{i1}\).
protein for which Kozasa et al. claimed to purify, via this method, an amount of 1400 μg from 600 mg of Sf9 membranes (Kozasa & Gilman 1995).

![Figure 2.3.7 Gβγ subunit purification PAGE](image)

Figure 2.3.7 Gβγ subunit purification PAGE: Coomassie Blue stained protein at approximately 37-39 kDa (shown by top arrow) indicates presence of Gβ4 and faint staining at 7kDa (shown by bottom arrow) indicates presence of Gγ2. Marker- Kaleidoscope Prestained standards (Bio-Rad). Lanes from left to right are aluminium fluoride elutions (1-6) which have separated the β4γ2 subunit from the Gαi1his subunit on the column, then the molecular weight (MW) standard.

The Gγ-subunit was not always as clearly visible as the co-purified Gβ subunit, the latter seen as a very light band (bottom arrow, Figure 2.3.7 at a molecular weight of approximately 7 kDa). This may have been due to less staining of the Gγ subunit due to the small molecular weight or slightly lower expression of the Gγ subunit (the differential staining could also be seen when the gamma subunit carried the hexahistidine tag).

The Gαq subunit was purified by combining it with the Gβ1γ2his dimer prior to loading onto the Ni-NTA beads. The Gαq subunit was purified using AlF4⁻ and the concentration was found to be 3.79 μM (0.157 mg/ml).

![Figure 2.3.8 Gαq subunit purification PAGE](image)

Figure 2.3.8 Gαq subunit purification PAGE: Coomassie Blue stained protein at approximately 45kDa (shown by arrow) indicates presence of Gαq. Marker- Kaleidoscope Prestained standards (Bio-Rad). Lanes from left to right are the aluminium fluoride eluted Gαq subunits, separated from the β4γ2His subunit captured on the column. Right lane, shows the MW standard.

Due to the slight increase in contaminating bands within the sample, western blot
analysis was used to confirm the presence of the \( G_\alpha_q \) subunit (Figure 2.3.9).

The \( G_\alpha \) antibody used in the western blot shown in Figure 2.3.9, was generated against an internal sequence of the \( G_\alpha \) subunit common to all \( G_\alpha \) subtypes, and therefore could recognise both the \( G_\alpha_1 \) and \( G_\alpha_q \) subtypes. Figure 2.3.9 shows that the \( G_\alpha_1 \) and \( G_\alpha_q \) subunits are present within the purified samples. It also shows that there is no non-specific binding to any proteins within the pure and non-pure \( \beta\gamma \) preparations in lanes 4 and 5. The \( G_\alpha_q \) purification yielded 3.79 \( \mu \)M purified protein (or 157 \( \mu \)g/ml). More than one band appears visible in the western blot analysis of both the \( G_\alpha_1\text{his} \) and \( G_\alpha_q \) proteins. These bands are unlikely to be non-specific binding due to the lack of their appearance in lane 5 (b) which has multiple non-specific proteins present within the sample. They are more likely to be degradation products of the \( G_\alpha \) protein. The higher band visible in lane 1 (b) could possibly display non-dissociated heterotrimer, which would migrate at around 85 kDa, a result which has previously been shown to occur (Silva 1991), or alternatively be the result of protein aggregation post-denaturation.
Chapter Two

2.3.4 Functional $[^{35}S]$-GTPγS reconstitution assays

Each receptor was tested for its ability to couple to and activate the G-proteins. This was monitored using the $[^{35}S]$-GTPγS binding assay (see Figure 2.3.10). This radionucleotide binding assay has been used extensively in GPCR functional studies (Windh & Manning 2002, McIntire et al. 2002, Gonzalez-Maeso et al. 2000, Panchalingam & Undie 2000, Fong et al. 1998, Jasper et al. 1998, Newman-Tancredi et al. 1998, Peltonen, Pihlavisto & Scheinin 1998, Pauwels et al. 1997, Lorenzen et al. 1993) and is effective for monitoring a fundamental process involved in GPCR signalling, i.e. the exchange of GDP for GTP on the activated Gα subunit. This portrays a true functional response as opposed to demonstrating only the ligand binding event.

Figure 2.3.10 $[^{35}S]$-GTPγS Functional Reconstitution Assay: Reconstitution of membrane preparations containing M2R; H1R or α2a-AR. 20 nM G-protein subunits, Gαi1 or Gαq and Gβγ or βγ2, were combined with receptor preparation; the indicated concentration of $[^{35}S]$-GTPγS; 10 μM AMP-PNP; 5 μM GDP. This reconstitution mix was then incubated with either buffer alone (basal, black column), an agonist (green column), or an agonist in the presence of excess antagonist (white column). a) 0.05 mg/ml H1-histamine receptor, 0.25 nM $[^{35}S]$-GTPγS used to monitor Gαi1 activation, stimulated with 1 mM histamine (agonist) and blocked with 100 μM pyrilamine (antagonist); n = 9. b) 0.05 mg/ml H1-histamine receptor, 0.25 nM $[^{35}S]$-GTPγS used to monitor Gαq activation, stimulated with 1 mM histamine (agonist) and blocked with 100 μM pyrilamine (antagonist); n = 3. c) 0.05 mg/ml M2-muscarinic receptor, 0.25 nM $[^{35}S]$-GTPγS used to monitor Gαi1 activation, stimulated with 120 mM carbachol (agonist) and blocked with 100 μM atropine (antagonist); n = 9. d) 0.1 mg/ml α2a-adrenergic receptor, 0.2 nM $[^{35}S]$-GTPγS used to monitor Gαi1 activation, stimulated with 10 μM adrenaline (agonist) and blocked with 100 μM yohimbine (antagonist); n = 2, representative data.

The H1-histamine receptor within Sf9 cell membrane extracts were capable of signalling
through the Gαq subunit (as shown in Figure 2.3.10b), which is the Gα subtype most commonly affiliated with H1R action in vivo (Raymond et al. 1991, Kuhn et al. 1996). Interestingly in this in vitro assay system it has also shown to signal through the Gαi1 subunit (Figure 2.3.10a). The relevance of this interaction with in vivo signalling has not been determined as it was not within the scope of this thesis, however, it has previously been reported that a functional H1R-Gαi1 interaction does occur in vivo (Seifert et al. 1994). The Gαi1 subunit was used in this study because it binds [35S]-GTPγS readily and specifically in this assay system. Both the M2R (Figure 2.3.10c) and α2aAR (Figure 2.3.10d) were also able to functionally couple to the Gαi1 subunit and induce the exchange of GDP for [35S]-GTPγS on the Gα in the presence of relevant agonists (carbachol for M2R and adrenaline for α2aAR). Specific activation was confirmed by the ability of an excess of antagonist (atropine for M2R and yohimbine for α2aAR) to block the agonist binding site and thereby inhibit activation of the Gα subunit.

2.4 Chapter summary

Functional H1-histamine receptors, M2-muscarinic receptors, and α2a-adrenergic receptors were successfully expressed in Sf9 insect cells using the baculovirus expression system. 3H-ligand binding and [35S]-GTPγS binding methods were used to confirm functionality of the receptors. Ligands which will be used throughout the thesis, both with and without fluorescent conjugates, were tested for their capacity to compete for 3H-ligand binding. This enabled the calculation of appropriate ligand concentrations to use in fluorescent ligand binding studies. Furthermore, functional G-proteins Gα1 +/- hexahistidine-tag, and the Gβ1 or Gβ4 dimerized with the Gγ2 +/- hexahistidine-tag, were successfully purified (as shown by SDS-PAGE in Figure 2.3.6-Figure 2.3.8, and by their activity validated using [35S]-GTPγS binding assays shown in Figure 2.3.10). This purification strategy underpins the protocols leading to the fluorescent labelling of the G-proteins used in the following chapter (chapter 3).
3 Monitoring Specific G-protein Interactions by Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET)

3.1 Introduction

This chapter introduces a fluorescent based method for measuring signal transduction for a functional GPCR:G-protein signalling complex. This assay platform was constructed in an attempt to replace the radioactive filtration based assay (described in section 1.5.2.2), with a homogeneous, fluorescence-based system, amenable to high-throughput screening applications. This chapter describes an assay developed using a donor and acceptor fluorophore on the G-protein $G_\alpha$ subunit and $G_\beta\gamma$ dimer. This assay design originally began as an undergraduate (honours) project I undertook, and this thesis expands on the viability and usability of the assay system. The chapter begins with an introduction to the fluorescence technique used in the assay (time-resolved fluorescence resonance energy transfer), as well as the fluorescent molecules and the conjugation technique used to label the G-proteins. The assay system previously established is then introduced. Results in this chapter confirm the capabilities of the G-protein interaction assay and further provide an indication of the feasibility to monitor G-protein binders and receptor-induced changes in G-protein interactions using the $\alpha_{2a}$-adrenergic receptor and the $M_2$-muscarinic receptor.

3.1.1 Fluorescence techniques in high-throughput screening

High-throughput screening (HTS) techniques have high importance in the area of drug discovery. The aim of HTS is to enable the testing of a large number of compounds for their activity as biological inhibitors or activators in an automated fashion. Screening technology is being adapted to also measure the functional properties of the potential drug candidate. This is favourable as it would decrease the number of steps required from lead compound discovery. Fluorescence techniques are popular for HTS due to the speed, level of detection, adaptability to homogeneous formats, and their applicability when using small volume samples. Some fluorescence techniques which are either already used in high-throughput screening systems, or are being developed to do so,
include fluorescence polarization, time-resolved fluorescence, fluorescence resonance energy transfer, time-resolved fluorescence resonance energy transfer (TR-FRET), and fluorescence fluctuations (Gomez-Hens & Aguilar-Caballos 2007). This chapter discusses the use of TR-FRET as a potential HTS assay technique for the study of GPCRs. In particular, the assay system was designed to provide a platform to determine the properties of the screened compound rather than simply its receptor binding capability. The strategy involves the use of TR-FRET to monitor the separation event of the G-protein heterotrimer, which is indicative of, and a generic marker for, receptor activation.

3.1.2 Protein labelling

There are a number of established and developing methods for the labelling of proteins with fluorescent molecules. These include, but are not limited to, the engineering of fluorescent proteins onto a protein of interest; chemical labelling using reactive fluorophores; and site directed labelling using affinity tags. The use of a fusion protein with fluorescent properties such as green fluorescent protein (GFP) and analogues thereof, requires the attachment to the host protein of a polypeptide which usually ranges in molecular mass from 22-36 kDa. Although many of these types of modifications have left the protein of interest functionally intact, there have been reports on the molecular limitations of the location of these tags on the proteins. This is evident in the case of the G\(\alpha\) subunit, for which the N and C-terminal regions are critical for its biological function. For this subunit, the fusion protein has most commonly been engineered into an internal loop of the G\(\alpha\) subunit (Janetopoulos, Jin & Devreotes 2001, Hughes et al. 2001, Yu & Rasenick 2002, Hynes et al. 2004). In addition to various fluorescent protein fusion strategies leading to the loss of function of a particular protein, there have also been reports of proteins gaining non-physiological functions, such as the G\(\gamma\) effector interactions reported by Zhou et al. (Zhou, Toth & Miller 2003). However, the use of fluorescent fusion proteins, has proven very useful for the monitoring of GPCR and G-protein interactions within the cell, and has provided information on GPCR localization, oligomerization, internalization, as well as G-protein localization and function (as reviewed in Hebert et al. (Hebert, Gales & Rebois 2006)).

Alternatively, labelling techniques using small organic fluorophores are also popular for
biochemical assays. Fluorophores with a large range of excitation and emission wavelengths are commercially available with reactive groups capable of labelling certain functional groups within the protein (see section 3.1.2.1). The advantages of the small fluorescent probes over fusion proteins primarily reside in their size (generally less than 1kD, which is less likely to interfere with protein function than e.g. a larger, protein fusion alternative). Furthermore, the ease of labelling (as opposed to undertaking the molecular biology processes necessary for protein fusions), also makes them an attractive option. With appropriate bioconjugation chemistries, these fluorophores can be directed to natural amino acids (usually cysteine or lysine residues) either at one or more native sites within the protein, or introduced via addition of mutations into specific sites within the protein (i.e. if there are no other amino acids of this type within the polypeptide). Alternative labelling techniques such as the use of unnatural amino acids (Kapanidis & Weiss 2002); quantum dots (Medintz et al. 2005); and site-directed chemical labelling (George et al. 2004, Chen et al. 2005, Keppler et al. 2004, Adams et al. 2002, Sculimbrene & Imperiali 2006, Kapanidis, Ebright & Ebright 2004, Marks & Nolan 2006, Soh 2008) offer a range of advantages over the fluorescent protein fusions and chemical labelling techniques described above.

### 3.1.2.1 Reactive fluorophores

Protein labelling techniques for commercially available fluorescent probes use a range of bio-conjugation reactive groups to covalently couple small fluorescent probes to biomolecules (a review of these strategies is available as a handbook from Molecular Probes, Invitrogen). Targeted reactive groups on proteins for labelling are most commonly primary amines (found on the lysine side chain, ε-amines, and N-terminal α-amines), and sulfhydryl groups (commonly found on reduced cysteines). Cysteines are generally less abundant than lysine groups within a protein sequence and therefore are commonly used for site-directed labelling protocols. One or more cysteines can be labelled in the native protein, or alternatively, site-directed mutagenesis can replace cysteine groups with another structurally similar amino acid, such as serine while introducing a single cysteine at a specific location (Kim et al. 2008). Alternatively, if the protein does not have any pre-existing cysteine residues, it is possible to introduce them where appropriate, as long as functionality of the protein is not altered.
Common thiol reactive functional groups are iodoacetamides and maleimides. Of these two, the maleimide functional group is the most widely used due to the higher specificity and efficiency of the coupling reaction (Kim et al. 2008). Availability of reduced, surface exposed cysteine residues, pH, incubation time and the presence of interfering buffer components, (such as reducing agents containing thiol groups for sulfhydryl reactive probes), are all factors which can affect labelling efficiency.

### 3.1.3 Fluorescence resonance energy transfer

Fluorescence Resonance Energy Transfer (FRET) is a well established technology for measuring at the molecular level changes in the distance between various moieties in biological systems. The FRET process relies on the distance-dependent transfer of energy from a donor fluorophore to an acceptor fluorophore. The transfer of energy from the donor fluorophore to the acceptor fluorophore depends on two main events. Firstly, the transfer relies on the overlap of the emission wavelength of the donor molecule and the excitation wavelength of the acceptor molecule. Secondly, the efficiency of the transfer also depends on the distance between the two fluorophores; (efficiency is proportional $1/R_o^6$, where $R_o$ is the distance at which half the energy is transferred, and depends on the spectral characteristics of the dye and their relative orientation) (Selvin 2000). Commonly used fluorophores in biological FRET systems include variations of the Green Fluorescent Protein fusion protein family. These fluorescent proteins are used to monitor intramolecular and intermolecular interactions (see Figure 3.1.1).
FRET techniques offer a versatile method for studying GPCR signalling and G-protein interactions. While measurements have been made under physiological conditions in cell based systems (Vilardaga et al. 2003), the technology could potentially be used in a cell-free system using purified, reconstituted signalling components. Indeed, cell-free studies of the kinetics of G-protein subunit association have been carried out using stop-flow kinetics in combination with a fluorescein-labelled $G_{\alpha}$ and an eosin-labelled $\beta\gamma$ dimer, the latter acting as a quencher upon association (Neubig, Connolly & Remmers 1994). In that study, the FRET data demonstrated that the kinetics of $G_{\alpha}$ and $\beta\gamma$ binding were relatively fast, with very high affinity, however, the level of signal (i.e. the quenching of fluorescence) was poor, and therefore would probably not be suitable for a robust high-throughput assay.

### 3.1.4 Spectrally matched fluorophores for FRET applications

One limitation of FRET techniques is the spectral cross-talk which occurs due to the relatively broad wavelength bands for the absorption and emission of fluorophores. These broad bands are due to solvent and environmental interactions and the presence of multiple vibrational levels of the ground and excited states (Dickson, Pollak & Diamandis 1995). This can increase the background signal level as the acceptor fluorophore can be excited by wavelengths used to excite the donor fluorophore.
Further problems arise due to the large background signal arising from contaminants in the reaction mixture which may also be excited by the excitation wavelength of the donor or the acceptor. Finally, the resonance energy transfer depends not only on the proximity of the fluorophores and the site and number of labels on a molecule, but also on probe orientation. Probes which can overcome the some of the abovementioned limitations utilize the unique luminescent properties associated with some of the ions within the lanthanide series.

### 3.1.4.1 Terbium chelate

Lanthanides (also called the rare-earth metals) have been shown to offer many advantages as donors in FRET as they overcome some of these problems referred to above, and this is primarily due to their inherent long emission lifetimes which allows time-resolved or time-gated measurements to be taken. Lanthanide ions alone only weakly absorb and emit electromagnetic radiation. For this reason they have been conjugated to various chelate ligands and antenna molecules which initiate and greatly amplify excitation; (for reviews of lanthanide properties see review by Hemmilä and Laitala (Hemmilä & Laitala 2005)). The Terbium cryptate conjugate used in the development of this assay system was DTPA (Diethylenetriaminepentaacetate)-cs124 (7-amino-4-methyl-2(1H)-quinolinone) (see Figure 3.1.2b). The organic acceptor used was Alexa546 (see Figure 3.1.2a) which has a high quantum yield and an excitation wavelength closely corresponding to the emission wavelength of the Terbium cryptate donor with this FRET pair exhibiting an $R_o$ of 50-60 Å (Selvin 2002).

![Fluorophore structures](image)

**Figure 3.1.2 Fluorophore structures.** a) Alexa 546 (maleimide) (Molecular Probes Inc.) b) DTPA (Diethylenetriaminepentaacetate)-cs124 (7-amino-4-methyl-2(1H)-quinolinone)Tb (Invitrogen™).

The chelate moiety serves several purposes: it provides a scaffold for covalently attaching the “antenna” molecule close to the lanthanide; it displaces water from the primary coordination sphere of the lanthanide, protecting the lanthanide from
quenching; and finally, it provides for attachment of a reactive group such as a maleimide or succinimidyl ester group for coupling to biomolecules such as proteins (Xiao & Selvin 2001).

Figure 3.1.3 Spectral overlap: Tb-DTPA-cs124 2nd emission peak (solid, blue); Alexa546 excitation peak (broken, red); grey band indicates wavelength at which Alexa546 emission (solid red) is measured, at a wavelength of 572nm, where terbium emission is negligible.

Spectral characteristics of the lanthanides which arise from the unusual nature of the atomic states have proven to be valuable in the application of resonance energy transfer (RET) experiments involving DNA (Li & Selvin 1997) and proteins (Heyduk 2001). The advantages of lanthanides such as europium and terbium over other fluorophores as donors in RET experiments include the microsecond to millisecond luminescent lifetime of the lanthanides, as opposed to fluorescent lifetimes (of other non-lanthanide fluorophores) of the order of 1-100 nanoseconds. Additionally, lanthanides exhibit a number of narrow, sharply defined, emission peaks as well as a large Stokes shift between absorption and emission wavelengths, both of which are favourable characteristics contributing to the significant increase in the signal-to-noise ratio compared with non-lanthanide based fluors.
Due to these long emission lifetimes (see Figure 3.1.4) the opportunity exists to delay (gate) the measurement of the FRET-induced fluorescence emission of the fluorophore. Directly excited acceptors and contaminants will decay to zero shortly after the excitation pulse because of their nanosecond lifetimes. Thus any sensitized emission will be the result of the energy transfer from the long lifetime emission of the lanthanide donor. This highly desirable feature of the lanthanide was expected to aid studies involving membrane extracts, which, due to high levels of turbidity and inclusion of any cell content associated with the membrane, could display significant background fluorescence. Recently, TR-FRET technology using a europium chelate donor and a cyanine fluorophore acceptor has been used in the development of a homogeneous assay for a specific protein-protein interaction essential for the initiation of transcription in eubacteria (Bergendahl, Heyduk & Burgess 2003). A homogeneous assay for sigma (transcription factor) binding to RNA polymerase based on fluorescence resonance energy transfer was developed by using europium-labelled σ70 and an IC-5-labelled fragment of the β` subunit of RNA polymerase. The study illustrated the use of TR-FRET to investigate protein-protein interactions in a homogeneous assay system. TR-FRET has also been used in the investigation of the dimerisation of the H1-histamine receptor in a cell-based assay system (Bakker et al. 2004). In the abovementioned study, antibodies to the FLAG or myc tags engineered on the expressed receptors, were conjugated to allophycocyanin and Eu³⁺ respectively.

Terbium and europium are the two most useful lanthanides for use as fluorescent probes. This is due to their spectral characteristics and emission intensities when incorporated in the appropriate chelator and excited with an adjoined antenna molecule.
Both have multiple emission peaks, but the peaks of europium are slightly red-shifted (which may reduce chances of adsorption of europium emission by assay components).

The main advantages of terbium as the lanthanide donor over the europium chelate is that terbium luminescence is quenched by hydroxyl groups to a lesser extent than europium once it is chelated, primarily due to the larger energy gap between the lowest excited level and the highest ground state (Hemmilä & Laitala 2005). Additionally, longer lifetimes and higher quantum yields of the terbium chelate probe in the presence of acceptor molecules when compared to that of the europium chelate have also been reported using three different chelates for the lanthanides (Xiao & Selvin 2001).

### 3.1.5 G-protein TR-FRET assay

In recent studies, high affinity G-protein interactions in solution have been shown using Time-Resolved Fluorescence Resonance Energy Transfer (Leifert et al. 2006). Below is an example of the current capabilities of the system (see Figure 3.1.5 - Figure 3.1.8). Once the G-proteins are fluorescently labelled via maleimide linkage to available cysteine residues, they maintain their ability to functionally couple to GPCRs as shown in the $[^{35}\text{S}]$-GTP$_\gamma$S reconstitution assay (Figure 3.1.5). The G$\alpha_1$His protein labelled with the acceptor fluorophore (Alexa 546) in the presence of the G$\beta_1\gamma_2$ dimer labelled with the terbium chelate, when reconstituted with the $\alpha_{2a}$-adrenergic receptor (AR), showed an increase in $[^{35}\text{S}]$-GTP$_\gamma$S binding in the presence of UK14304 (a stable $\alpha_{2a}$-AR agonist). This signal was subsequently blocked when an antagonist, yohimbine was used in the assay. The fold increase of stimulation (~5.5 fold) generated in the absence of labels (Figure 3.1.5a) is very similar to that obtained in the presence of labelled protein (Figure 3.1.5b) (Leifert et al. 2006) suggesting that the small fluorescent probes do not alter the functional properties of the G-protein subunits.
The high affinity interaction of the G-protein subunits was demonstrated by the increase in the TR-FRET signal generated at the emission wavelength of the acceptor probe after excitation at the donor excitation wavelength. As discussed in 3.1.4.1, the large bandwidth of the excitation and emission of the terbium chelate donor, eliminates direct excitation of the Alexa acceptor by the 340 nm excitation wavelength of the terbium chelate. The terbium chelate itself, however, does have a small emission at the 572 nm emission wavelength of the acceptor Alexa 546 fluorophore, as shown by the nearly horizontal line of the $\alpha_2$His-Tb conjugate (\(\nabla\)) shown in Figure 3.1.6. When $\alpha_2$His-Tb is in the presence of $\beta_1\gamma_2$ Alexa, fluorescence increases ~7.6 fold above background (Figure 3.1.6). This increase in fluorescence at the acceptor wavelength indicates that the terbium and Alexa fluoros present on the protein binding partners are in close enough proximity for efficient energy transfer from donor to acceptor. This increase in TR-FRET fluorescence indicates that the subunits carrying the donor and acceptor fluoros are binding to one another, creating the heterotrimeric G-protein.
Figure 3.1.6 Time Resolved Resonance Energy Transfer between Gα and βγ. Time Resolved Fluorescence Resonance Energy Transfer between 50 nM Gαi1HisTb + 50 nM βγ2Alexa (■). The background emission of the Terbium labeled subunit alone (▲). The excitation and emission filters used were 340 nm and 570 nm, respectively. Following a 50 μs delay, emission data were collected for a period of 900 μs. Dilutions of G-proteins were made in a buffer of 50 mM TRIS, 10 mM MgCl₂, 50 mM NaCl and 1 mM dithiothreitol, pH 8.0 (data are mean ± SEM, n=3). a.u. arbitrary units.

This TR-FRET signal indicated that the Gα and Gβγ subunits were in close proximity, and from previous experience, we can assume that this is an indication of heterotrimer formation. To show that this was indeed the case, the heterotrimer labelled with the donor and acceptor fluorphores were exposed to various concentrations of an unlabelled binding partner (Gα) to compete for Gβγ Alexa546 binding with the labelled GαTb in solution (Figure 3.1.7a). This resulted in a concentration dependent decrease in the TR-FRET signal measured at the acceptor emission wavelength of 572nm. This decrease was also observed when the opposite binding partner, the Gβγ dimer was used as the competitor at an excess concentration (Figure 3.1.7b) (Leifert et al. 2006).
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Figure 3.1.7 Unlabelled binding partners compete for binding to G\textsubscript{ai1} or G\textsubscript{b/\gamma 2}, causing a decrease in TR-FRET. (a) TR-FRET between 50 nM G\textsubscript{ai1}HisTb + 50 nM G\textsubscript{b/\gamma 2}Alexa was reduced in a dose dependent manner upon addition of increasing amounts of unlabeled G\textsubscript{ai1} over a time period of 20 min after prior association of the subunits occurred over the first 10 minutes. (b) Addition of a 10-fold excess of unlabeled G\textsubscript{b/\gamma 2} (final concentration of 200 nM) caused a 70% reduction in TR-FRET between 20 nM G\textsubscript{ai1}HisTb + 20 nM G\textsubscript{b/\gamma 2}Alexa. Data shown are mean ± SEM, (n=3). Background terbium emission at 572 nm ranges from 2500 a.u. to 1200 a.u. over 20 min and has been deducted. (c) TR-FRET between 50 nM G\textsubscript{ai1}HisTb + 50 nM G\textsubscript{b/\gamma 2}Alexa was reduced in a dose dependent manner upon addition of increasing amounts of unlabelled G\textsubscript{ai1}, with an IC\textsubscript{50} of 54.5 nM ± 19.3 nM. Data are mean ± SEM, n= 3. (Leifert et al. 2006).

The equilibrium dissociation constant $K_i$ (described in section 2.2.2.7), generally describes the affinity between receptor and ligand, measured using competition binding studies. Here the affinity between G-protein subunits is measured using a similar approach. Here we can demonstrate that G\textsubscript{\alpha} subunits labelled with the fluorescent conjugate show similar affinities for the \beta\gamma subunit as non-labelled G\textsubscript{\alpha} subunits. The competitor in this case is the unlabelled G\textsubscript{\alpha} subunit and the EC\textsubscript{50} value obtained in the competition assay was 54.5 nM (Figure 3.1.7c). Using the formula mentioned in the second chapter of this thesis (section 2.2.2.7), and the $K_d$ value obtained for this interacting couple, of 2.4 nM (Leifert et al. 2006), the $K_i$ value of the non-labelled G\textsubscript{\alpha} subunit is calculated to be 2.5 nM, almost identical to the labelled G\textsubscript{\alpha} subunit.

An additional challenge was to determine whether the TR-FRET assay format could be
used to monitor the dissociation of the activated G-protein heterotrimeric complex. This has important implications as an additional aim was to monitor receptor induced G-protein activation (and thus dissociation). To do this, aluminium fluoride was used as it is known to dissociate the Gα subunits from the Gβγ subunits (in fact this very principle was utilised during the purification of the G-proteins from the Ni-NTA columns as shown in section 2.3.3). Aluminium fluoride, which is formed by the addition of 10 mM NaF and 30 μM AlCl₃, is capable of simulating the GTP bound configuration of the Gα subunit by creating a group similar to that of the third phosphate of GTP, on the Gα-bound GDP molecule (Gilman 1987). Following addition of AlF₄⁻, a decrease in fluorescence of the acceptor emission at 572nm was observed (Figure 3.1.8), indicating a likely separation (dissociation) of the Gα subunits from the Gβγ subunits.

**Figure 3.1.8 AlF₄⁻ decreases G-protein affinity.** Time Resolved Resonance Energy Transfer between 15 nM Gα₁hislTb + 15 nM β₂γ²Alexa. The excitation and emission filters used were 340 nm and 570 nm, respectively. Following a 50 μs delay, emission data were collected for a period of 900 μs. G-protein samples were diluted in TMND (50 mM TRIS pH 8.0, 10 mM MgCl₂, 50 mM NaCl and 1 mM dithiothreitol) to a final volume of 100 μl. 10 mM NaF and 30 μM AlCl₃ were added after 7 minutes to initiate dissociation of subunits. Representative data.

The fluorescence does not decrease completely to background once the Gα is activated using AlF₄⁻. This has been attributed to residual affinity of AlF₄⁻ bound Gα subunits for Gβγ (Leifert et al. 2006), which has been reported previously (Sarvazyan, Remmers & Neubig 1998). Alternatively, an incomplete saturation of the Gα subunits with AlF₄⁻ leaves some inactive Gα₁Tb subunits in the assay, able to continue residing in the heterotrimer form (Leifert et al. 2006).
3.1.6 Assay development and assessment

The TR-FRET G-protein assay discussed in section 3.1.5 displayed some promising results showing that under the assay conditions used, both heterotrimer formation and separation in vitro, could be determined. Rigorous studies were still necessary to further elucidate the viability of the assay, including contributing artefacts in the system, reliability of assay format (Z’-factor), and adaptability of the G-protein assay to couple to a receptor based assay system.

3.1.6.1 Z’-factor

Assay quality has generally been based on the signal to noise ratio of the system. In the late 90’s Zhang et al. described a screening window coefficient in the context of high throughput assay screening (Zhang, Chung & Oldenburg 1999). The coefficient, termed the Z’-factor, takes into account both the dynamic range of the assay signal as well as the variation in the data obtained from the assay signal. These two components are not addressed when monitoring the signal-to-noise ratio alone. The Z’-factor provides a tool for comparison and evaluation of the quality of an assay and is now universally accepted and used in screening programs worldwide.

3.2 Materials and methods

3.2.1 Materials

For baculovirus stock origins, please refer to section 2.2.1 in the previous chapter. The hexahistidine-tagged RGS4 protein, expressed in E. coli and purified using IMAC, was generously provided by Tamara Cooper (CSIRO). The recombinant pQE60 vector containing RGS4 was provided by Professor R. Neubig (University of Michigan). Tachyplesin and the test peptide used in the TR-FRET competition assays (Gly-Ala-Glu-Gly-Cys-Pro-Gly-Cys-Cys-Gly-Gly-Gly) were prepared by AusPep, Australia. FITC labelled tachyplesin was provided by Dr. Wayne Leifert (CSIRO). The fluorescent probes Alexa fluor 546 C5 and cs124-DTPA-EMCH-terbium were purchased from Invitrogen (Victoria, Australia). GTPγS, [35S]-GTPγS and all other reagents of the highest quality grade were purchased from Sigma-Aldrich (NSW, Australia).
3.2.2 Methods

3.2.2.1 Fluorophore labelling and purification of G-proteins

G-proteins and GPCRs were expressed and prepared as described in section 2.3.2.2. The fluorophore labelling procedure was carried out while G-proteins were loaded onto a nickel affinity column as described previously (Leifert et al. 2006). For labelling procedures, the β-mercaptoethanol was removed to eliminate interference in thiol conjugation when using a maleimide linker, by washing with 50 ml buffer A (Table 2.2.1-1) without the β-mercaptoethanol present. The Alexa 546 maleimide dye was solubilized in anhydrous DMSO to a concentration of 4.8 mM and the terbium cryptate maleimide conjugate in HEPES (pH 8.0) made to a concentration of 1.09 mM and added at an approximate 5x molar excess over G-proteins. The columns were incubated at room temperature (RT) for 3 hr (terbium) or 2-3 hr (Alexa) as indicated by manufacturer’s instructions. Following incubation, the column containing the labelled hexahistidine-tagged G-proteins was washed with 50 ml buffer A (Table 2.2.1-1) containing 5 mM imidazole and 300 mM NaCl, pH 8 to remove unbound Alexa until the eluent containing non-conjugated fluorescent probes produced fluorescence similar to that of buffer alone. Fluorescence was monitored by measuring the emission at 550 nm for the terbium conjugate, and 572 nm for the Alexa 546 fluorophore after excitation at 340nm or 545nm, respectively (HITACHI Fluorescence Spectrophotometer 650-10S). This was used to verify that the protein on the column had been washed thoroughly to remove any non-conjugated fluorescent probe. The non-hexahistidine-tagged G-protein subunits were eluted with 400 μl fractions of buffer E (Table 2.2.1-1) containing 10 mM NaF and 30 μM AlCl3 (AlF4-). The remaining hexahistidine-tagged G-protein subunits were eluted from the nickel column with buffer E containing 150 mM imidazole. Aliquots of elution fractions were analysed using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Elution fractions containing the appropriate G-protein subunit were pooled (to ≤ 3ml) and dialysed with 4 changes each of 200 ml buffer F, then overnight against 200 ml buffer F (Table 2.2.1-1) using a Slide-a-lyzer (Pierce Chemical Company). Following dialysis, the G-protein subunits were snap frozen in liquid N2 and stored at -80°C in buffer F or in 50% (v/v) glycerol in buffer F. Final protein concentration was determined by SDS-PAGE of G-protein subunits (using BSA Standards), followed by laser scanning densitometry (LKB
ULTOSCAN XL, Enhanced Laser Densitometer, Sweden), or Bradford Protein Assay (Bradford 1976). The yields of RGS4 were approximately 3 mg/ml of bacterial culture, while those of the G-proteins ranged from 0.1-1.0 μg/ml of Sf9 insect cell culture. Purity was routinely >95%.

3.2.2.2 [35S]-GTPγS reconstitution assay

Functionality of G-proteins was measured using a modified protocol of the well established [35S]-GTPγS binding techniques (Windh & Manning 2002). This method was described in section 2.2.2.8.

3.2.2.3 [35S]-GTPγS binding assay in the absence of receptor

Assays used to determine [35S]-GTPγS binding to the Gα subunit independent of receptor stimulated activation were carried out in 100 μl volumes of TMND. To follow the location of the Gα subunit in relation to the Gβγ dimer post incubation with the [35S]-GTPγS nucleotide, the assays were conducted in the presence of a Ni-NTA bead solution, which could specifically capture the hexahistidine-tagged subunit. Assay filtration was carried out over a filter stack (as previously described (Leifert et al. 2005b)) comprising a GF/C filter beneath a paper filter. The Ni-NTA beads and associated proteins are filtered over a paper/GFC stack followed by 3 x 4ml washes with TMN buffer. The paper filters capture the relatively large beads (approximately 45-165 μm in diameter) and any associated proteins, while any proteins which are not associated with the bead can be washed through and captured on the GFC filter.

3.2.2.4 Labelling efficiency

Labelling efficiency was determined by comparing the fluorescence emission intensity of Alexa or terbium in solution against G-protein conjugated label (subtracting any background fluorescence for unlabeled protein). Calibration curves of terbium label and Alexa 546 in solution were generated using a Victor³ plate reader (Perkin Elmer Corp.) at excitation and emission wavelengths of 340/545 nm and 545/572 nm, respectively.

3.2.2.5 TR-FRET assays

Fluorescence measurements were obtained using a Victor³ (Perkin Elmer Corp.)
multilabel plate counter fitted with a 1500V Xeon flash light source. Experiments were carried out in black 96-well plates. The required amounts of proteins labelled with acceptor or donor fluors were aliquotted onto either side of a well so that mixing did not occur until required. Addition and mixing of TMND buffer into the well to give a final assay volume of 100 μl, commenced the reaction. To utilise the long-emission lifetime of the Tb chelate donor and the time-resolved emission of the acceptor fluorophore, the measurement was gated for a short time period to minimise background emission from the assay components contributing to this signal. TR-FRET values were measured using the following instrument settings; excitation 340 nm, emission 572 nm, 50 μs delay, and 900 μs counting duration (unless otherwise stated). Readings were taken until the fluorescence reading stabilized. If required, after stabilization of the fluorescence reading, other components such as unlabelled Gα11His, RGS4, or Gβγ, mastoparan, tachyplesin, were added and fluorescence readings then continued until fluorescence again stabilized. Experiments using AlF4– were conducted by dispensing TMND buffer containing 10 mM NaF and 30 μM AlCl3. Background fluorescence was determined by the addition of the appropriate concentration of terbium labelled protein to TMND buffer and exposure to the same TR-FRET conditions.

3.2.2.6 TR-FRET reconstitution experiments with labelled G-proteins and receptor

TR-FRET assays were carried out using a similar method to that described in section 3.2.6. The receptor was included into the assay in two forms, the crude membrane extract, or the extruded membrane extract (see section 3.2.2.7). G-protein subunits labelled with spectrally matched fluors (Tb & Alexa) were reconstituted with membrane extracts containing recombinant GPCRs. GDP concentrations were equivalent to that carried over from buffer F (dialysis buffer) into the assay, and GTPγS was added to the assay as indicated. Plate reader parameters included an excitation wavelength of 340 nm and emission wavelengths of 545 nm (donor emission) and 572 nm (acceptor emission) and a delay time of 100 μs. Readings were taken to calculate the net using the following relationship:

Net FRET = Fluorescence @ 572nm
Fluorescence @ 545nm
3.2.2.7 Extruding GPCR membrane extract

Extrusion of lipid preparations is commonly used to create lipid vesicles by forcing the lipid solution through pores of equal diameter at high pressure. The process commonly results in an opaque lipid solution extruded to a clearer solution of liposomes. This is due to the relationship between turbidity of a solution (measured by optical density) and particle size (Pozharski, McWilliams & MacDonald 2001). Extrusion is used here in an attempt to decrease optical interferences in the TR-FRET assay. *Sf9* membrane preparations were diluted to a total protein concentration of 0.75 mg/ml. Following this some preparations were then extruded through a 1 μm polycarbonate membrane (Whatman trach-etch) followed by a 400 nm polycarbonate membrane (Whatman trach-etch) at room temperature.

3.2.2.8 Z′-Factor calculations

To determine the Z′-factor, the heterotrimer TR-FRET assay was performed with functional subunits (positive control) and with denatured subunits (negative control). The means and standard deviation of the means were determined using the statistics function in Prism™ software. The standard deviations of the mean were added together (termed SSD) and the Z′-factor calculated using the following formula: $Z'\text{-factor} = 1.0 - (3.0 \times \text{SSD}/R)$, where $R$ (dynamic range) is the difference between the mean signal from the positive control and the mean signal from the negative control (Zhang, Chung & Oldenburg 1999).

3.2.2.9 Fluorescence microscopy

Fluorescence Microscopy was used to visualise specific capture of hexahistidine-tagged G-proteins and subsequent capture of fluorescently labelled, interacting partners. An Olympus BX41 fluorescent microscope fitted with FITC and Cy3 filters was used. Images were obtained using a 20x UPlanFl objective (NA 0.5), and SPOT Advanced image capture software (Diganostic Instruments, Inc.). Beads were pre-incubated with hexahistidine-tagged proteins ($G\alpha_{1HIs}$, 2.5 μM, $\beta_1\gamma_{2HIs}$, 2.85 μM) or buffer, then washed. The washing procedure included dilution of beads into buffer, centrifuging the beads, removing supernatant, and replacing with fresh buffer (repeated 3 times). The beads were then incubated with fluorescent Tachyplesin (FITC labelled) or non-
hexahistidine-tagged $\beta_4\gamma_2$ (Alexa546 labelled) and washed as described above. Beads were pipetted onto a glass microscope slide with a coverslip for fluorescent microscopy.

### 3.2.3 Data analysis

Data was analysed using Prism™ (GraphPad Software Inc., San Diego CA, USA). Data shown is mean ± SEM where sample number (n) is greater than or equal to 3, however, if an experiment was conducted in duplicate, data represents mean ± range of duplicates. Where error bars are not visible, they are within the data point symbol (unless otherwise stated). Statistical analysis (Student’s t-test) was performed using Prism™.

### 3.3 Results and discussion

#### 3.3.1 Fluorescently labelled G-protein activity

The functionality of all preparations of labelled and purified G-proteins (as shown by fluorescent properties and SDS-PAGE, respectively), was routinely monitored using the [$^{[35S]}$-GTP$\gamma$S assay. A representative example of functional labelled G-protein subunits was demonstrated in the introduction to this chapter, (Figure 3.1.5). In this case, signalling activity of G-proteins labelled with terbium or Alexa 546 was measured by specific binding of [$^{[35S]}$-GTP$\gamma$S (final concentration 0.25 nM) induced by the specific activation of the $\alpha_{2A}$AR with the agonist 10 $\mu$M UK14304 in the presence of 20 nM of labeled G-proteins. [$^{[35S]}$-GTP$\gamma$S binding for labelled proteins increased from approximately 0.02 fmol (basal) to 0.14 fmol (stimulated) (Figure 3.1.5), reflecting the GDP-GTP exchange following the activation of $G_{\alpha_i}^{1_His}$ by the agonist bound receptor. To show specificity of stimulation, the $\alpha_{2A}$AR antagonist yohimbine was used to compete for binding of UK-14304 agonist to the $\alpha_{2A}$adrenergic receptor and no stimulation of the $G_{\alpha}$ subunit above basal was evident.

Terbium labelling efficiency of Tb:G-protein ranged from 1:4 to 1.6:1 (label:protein). Alexa546 labelling efficiency ranged from 1:4 to 1.2:1 (label:protein) for functional, labelled G-proteins, as reported previously (Leifert et al. 2006). $G_{\alpha_1}$, $G_{\beta_3}$ and $G_{\gamma_2}$ subunits contain 8, 14 and 1 cysteine residue, respectively (Wall et al. 1995), however not all of these residues are in a suitable position or orientation for conjugation to occur.
Yang et al. have previously reported that the labelling of a G\(\alpha\) subunit with lucifer yellow via thiol linkage occurred predominantly on two cysteine residues, Cys\(^{210}\) and Cys\(^{347}\) located in the switch II region and the C-terminus, respectively (Yang et al. 1999). Two surface exposed cysteine residues are also present within the G\(\alpha_{11}\) subunit (as determined by the space-filled model from the protein databank [PDB:1GP2] (Berman et al. 2000)). These are the Cys\(^{305}\) and Cys\(^{325}\) residues, however, the latter may have slightly limited accessibility. An additional Cysteine (Cys\(^{214}\)), is present within the G\(\alpha\)/G\(\beta\gamma\) interface which is not available as labelling is carried out on the bound heterotrimer. The G\(\beta_1\) subunit appears to have only one accessible cysteine residue, Cys\(^{271}\), with perhaps limited accessibility to Cys\(^{204}\) and Cys\(^{25}\). Five of the cysteine residues on the G\(\beta\) subunit are present in regions that are included in the hydrophobic core of the G\(\alpha\)/G\(\beta\gamma\) interface and are not likely to be accessible for fluor labelling. The G\(\beta_4\) subtype has 90% sequence homology to the G\(\beta_1\) subunit and all but one of the cysteine residues are spatially conserved (Cys\(^{233}\) of G\(\beta_1\) is not present in G\(\beta_4\)).

Labelling efficiencies differed between batches primarily due inability to deduce protein concentration prior to the labelling. This occurred due to the protocol with which the G-proteins were fluorescently labelled. Labelling of the G-proteins was carried out prior to being eluted from the purification column. This was done in order to label the proteins in the heterotrimer form (so as to not label residues within the subunit binding interface), whilst enabling the separation of the G-protein subunits (G\(\alpha\) and G\(\beta\gamma\)) during purification to result in pure G\(\alpha\) or G\(\beta\gamma\). Instead of calculating the G-protein concentration prior to labelling, the concentration was extrapolated from previous purifications from similar infections, which consequently introduced variation in molar ratios of protein to dye. Additionally, using the thiol reactive probes introduces problems with labelling efficiency due potentially to the oxidation of the thiols back to disulfides during the process in which the reducing agent was removed prior to the introduction of the maleimide probes. This in turn reduces the number of cysteine residues available for labelling. Although these proteins showed promise in TR-FRET assays described here, further investigation (prevented by time-limitations) into alternative protein labelling strategies may aid in standardising the labelling efficiency and limiting variation between subunits prepared separately; (a project investigated by Tamara Cooper in our laboratory).
3.3.2 Characterisation of G-protein TR-FRET Assay

The TR-FRET interaction between the G-protein subunits had been shown to decrease in the presence of an excess of an unlabelled G-protein subunit binding partner, and in the presence of the Gα activator, aluminium fluoride (as described in section 3.1.5). These results provided evidence of the specificity of the G-protein interaction, however, further investigation was undertaken to determine if there was any contribution of non-specific FRET. Firstly, experiments were conducted to examine the effect of free acceptor fluorophore in solution when in the presence of the terbium labelled protein. This was carried out in an attempt to determine whether diffusional collisions were contributing to the TR-FRET signal that was being measured. This non-specific interaction has previously been observed in cell-based FRET systems. Within the cell, FRET artefacts could be amplified due to restricted degrees of freedom of the labelled proteins within the cell membrane or within a cellular compartment. Mercier et al. used the term “bystander BRET” to describe the non-specific interactions observed in GPCR dimerization studies of cell membranes using bioluminescence resonance energy transfer (Mercier et al. 2002). Results shown in Figure 3.3.1 indicate that artefacts arising from the production of “bystander” FRET, occur at donor:acceptor ratios (D:A) $\geq 1:60$. The D:A ratios used in the G-protein TR-FRET assays are much lower. Additionally, stringent washing conditions ensured the removal of unbound fluor after labelling reactions.
Figure 3.3.1 Contribution of bystander TR-FRET. Steady state TR-FRET between 500 pM labelled GβγTb + GαiplHisAlexa (labelled protein concentration indicated on x-axis) (■). TR-FRET between 10 nM GβγTb (500 pM labeled G-protein) and Alexa 546 free in solution i.e. "Bystander FRET" (▲). Data shown are mean ± SEM, (n=3). D:A, donor:acceptor ratio.

3.3.3 Z'-factor

Further confirmation that it was indeed the specific G-protein interaction that was producing the FRET response, was obtained by the investigation of TR-FRET between a functional donor labelled subunit and a proteolytically degraded acceptor labelled protein binding partner (Figure 3.3.2). G-protein interactions were inhibited by the destruction of the subunit binding interface by protease treatment of a single subunit. Protease degraded protein subunits were shown to be inactive in 35S-GTPγS functional assays (assays described in section 2.2.2.8). The Z'-factor function requires the statistical analysis of both the positive control of the assay and the negative control. In this case the positive control was the TR-FRET signal at equilibrium upon heterotrimer formation between the donor labelled Gβγ subunit and the acceptor labelled Gα subunit. The negative control was the diminished TR-FRET signal which resulted when a binding partner within the system is replaced by its inactive form (degraded via protease treatment).
The general guidelines for determining assay quality using the Z'-factor state that a Z'-factor of 1 is ideal. This value is approached when you have a large dynamic range and very small standard deviations. A Z'-factor between 0.5 and 1 is considered excellent, a value less than 0.5 but above 0 is considered marginal while a factor below 0 means that the signals from the positive and negative controls overlap. While the sample size for the controls used here to calculate Z'-factor is not large, the calculated Z'-factor is 0.59, which means it lies within a range applicable for a reliable assay that could be used for screening purposes.

3.3.4 GTPγS binding to Gα subunit in the absence of receptor

The interaction of G-proteins with guanine nucleotides is well established in the area of G-protein research. During the establishment of the TR-FRET G-protein interaction assay, experiments were conducted using various concentrations of GDP to ensure that the heterotrimer maintained an inactive conformation. However, the carry over of the 1 μM concentration of GDP present in buffer F (used for dialysis) was apparently adequate to occupy all GDP binding sites of the Gα subunits. This was concluded on the basis that there was little effect of GDP addition on the TR-FRET signal (data not shown).

As previously discussed, the GTP molecule becomes bound to the Gα subunit once the G-protein heterotrimer is activated by the coupled receptor. The GTP is hydrolysed by
the intrinsic GTPase activity of the \( \Gamma \alpha \) subunit, reducing the GTP to the GDP molecule. The GTP\( \gamma \)S molecule is the non-hydrolysable form of the nucleotide. Consequently, the role of GTP was tested as an activator of G-proteins in the absence of receptor.

![Figure 3.3.3](image-url)  
**Figure 3.3.3** GTP\( \gamma \)S does not appear to completely separate G-protein heterotrimer in the absence of receptor: Net FRET between 5 nM \( \Gamma \alpha _{i1} \)Tb and 15 nM labelled \( \Gamma \beta \gamma \) Alexa. The assay was carried out in 50 µl of TMND in 384-well black plates <10 minutes after addition. Steady state TR-FRET resulting from heterotrimer formation (black column) does not differ significantly from the TR-FRET in the presence of 100 µM GTP\( \gamma \)S (white column). Excitation = 340 nm and Emission = 545 nm and 572 nm after 50 µs delay and 900 µs count time. Data represents mean ± SEM, n = 24.

The small change in FRET fluorescence (measured at 572nm) shown in Figure 3.3.3 is not statistically significant (as calculated by unpaired t-test, GraphPad, PRISM). This result indicates that the \( \Gamma \alpha _{i1} \) protein labelled with the terbium cryptate donor molecule does not separate from the \( \Gamma \beta _{4} \gamma _{2} \) subunit labelled with an Alexa 546 conjugate, in the presence of GTP\( \gamma \)S. Alternatively it may indicate that the majority of the subunits do not separate or rearrange to an extent great enough to induce a decrease in the average FRET fluorescence of the system. Additional experiments were conducted over a longer incubation period and there also showed little change in FRET fluorescence. The rate of nucleotide exchange of GTP for GDP on the \( \Gamma \alpha \) subunit is a rate limiting factor in this reaction and is Mg\( ^{2+} \) dependent, as shown much earlier by Higashijima *et al.* (Higashijima *et al.* 1987). However, with 10 mM MgCl\( _{2} \) present in the TMND buffer used in this assay, this should not limit the nucleotide exchange. In experiments reported by Heithier *et al.*, FRET measurements between the affinity purified \( \Gamma \alpha _{o} \) and \( \Gamma \beta \gamma \) subunits labelled with fluorescein and rhodamine, respectively, indicate that a GTP\( \gamma \)S bound form of the \( \Gamma \alpha _{o} \) subunit is still able to associate with \( \Gamma \beta \gamma \), demonstrating that the complete dissociation of the heterotrimer does not necessarily accompany activation in solution (Heithier *et al.* 1992). Studies from Ganpat *et al.* and Yi *et al.* have also suggested an association/dissociation equilibrium in activated GTP\( \gamma \)S bound \( \Gamma \alpha \) and \( \Gamma \beta \gamma \) (Ganpat *et al.* 2000, Yi *et al.* 2003). These results lead to speculation that GTP alone is insufficient to activate the \( \Gamma \alpha \) subunit and the presence of the receptor
may be required to initiate complete activation and subsequent dissociation or rearrangement with the G\(\beta\gamma\) dimer. There is also evidence \textit{in vivo}, that the G-protein, specifically the G\(\alpha_i\) subunit, may rearrange upon agonist activation, rather than undergo complete dissociation from the other subunits in the heterotrimer (Bunemann, Frank & Lohse 2003). However, the negative cooperativity between G\(\beta\gamma\) and GTP\(\gamma\)S for binding on the G\(\alpha\) subunit, and hence the separation of the subunits, has been reported for a number of years (Gilman 1987), and it is more likely a result of differing assay conditions as to why these particular G-protein subunits do not show a significant decrease in the TR-FRET signal upon GTP\(\gamma\)S addition.

Preliminary results from the \([^{35}\text{S}]-\text{GTP}\gamma\)S assay using the same assay conditions also indicate, that despite the presence of GTP\(\gamma\)S, in the absence of receptor, many of the G\(\alpha\) subunits remain bound to the G\(\beta\gamma\) dimer. This is demonstrated by the binding of the G\(\alpha_i\) subunit onto Ni-NTA beads (monitored by \([^{35}\text{S}]-\text{GTP}\gamma\)S binding) captured by the hexahistidine-tagged \(\beta_1\gamma_2\)His subunit (Figure 3.3.4), and the subsequent high level of \([^{35}\text{S}]-\text{GTP}\gamma\)S which remain on the beads (captured on paper filters) after incubation of the radionucleotide with the heterotrimer.

\begin{figure}
\centering
\begin{subfigure}{0.45\textwidth}
\centering
\includegraphics[width=\textwidth]{figure_a}
\caption{a) 40 nM G\(\alpha_i\) + 40 nM \(\beta_1\gamma_2\)His or 40 nM G\(\alpha_i\)His were pre-incubated with 200 \(\mu\)l Ni-NTA beads. Beads were washed 3 x with TMN (beads spun down and supernatant removed) and then incubated with 0.9 nM \([^{35}\text{S}]-\text{GTP}\gamma\)S in 700 \(\mu\)l TMND for 90 minute at 28\(^\circ\)C. b) 40 nM G\(\alpha_i\) or 40 nM G\(\alpha_i\)His were incubated with 0.9 nM \([^{35}\text{S}]-\text{GTP}\gamma\)S in 700 \(\mu\)l TMND for 90 minutes at 28\(^\circ\)C. \([^{35}\text{S}]-\text{GTP}\gamma\)S binding was followed by running 100 \(\mu\)l assay mixtures over a filter stack composed of a paper filter capable of capturing beads (and associated proteins) on top of a GFC filter capable of capturing non-bead bound proteins (but not small molecules such as the \([^{35}\text{S}]-\text{GTP}\gamma\)S). Filters were washed 3 x 4ml TMN buffer. Values represent mean \(\pm\) SEM (\([^{35}\text{S}]-\text{GTP}\gamma\)S bound (fmoles)), n = 3.}
\end{subfigure}
\begin{subfigure}{0.45\textwidth}
\centering
\includegraphics[width=\textwidth]{figure_b}
\caption{b) 40 nM G\(\alpha_i\) + 40 nM \(\beta_1\gamma_2\)His or 40 nM G\(\alpha_i\)His were incubated with 0.9 nM \([^{35}\text{S}]-\text{GTP}\gamma\)S in 700 \(\mu\)l TMND for 90 minute at 28\(^\circ\)C. \([^{35}\text{S}]-\text{GTP}\gamma\)S binding was followed by running 100 \(\mu\)l assay mixtures over a filter stack composed of a paper filter capable of capturing beads (and associated proteins) on top of a GFC filter capable of capturing non-bead bound proteins (but not small molecules such as the \([^{35}\text{S}]-\text{GTP}\gamma\)S). Filters were washed 3 x 4ml TMN buffer. Values represent mean \(\pm\) SEM (\([^{35}\text{S}]-\text{GTP}\gamma\)S bound (fmoles)), n = 3.}
\end{subfigure}
\caption{\([^{35}\text{S}]-\text{GTP}\gamma\)S does not completely separate the G-protein heterotrimer in the absence of receptor:}
\end{figure}

The \([^{35}\text{S}]-\text{GTP}\gamma\)S binding assay was carried out using hexahistidine-tagged gamma subunits to capture the entire heterotrimer onto a Ni-NTA bead. The results indicate that the GTP bound form of the G\(\alpha\)-subunit is found most predominantly on the bead,
specifically bound via the hexahistidine-tagged $\beta_{12}$ subunit. Results here show radioactivity ($[^{35}S]\text{-GTP}\gamma S$ bound) of paper and GFC filters of $G\alpha_{11} + \beta_1\gamma_2\text{His}$ bound to a Ni-NTA bead (Figure 3.3.4a [left]), as well as the radioactivity of $G\alpha_{11}$ alone on paper and GFC filters (Figure 3.3.4b [left])$^1$. The amount of $[^{35}S]\text{-GTP}\gamma S$ which remains attached to the bead and is subsequently captured on the paper filter is $>10x$ that which is captured on the GFC filter presumably bound to separated $G\alpha_{11}$ subunits. However, when the $G\alpha_{11}$ subunit alone is washed through the same filtration setup, the higher $[^{35}S]\text{-GTP}\gamma S$ radioactivity is found on the GFC filter. The presence of the hexahistidine tagged subunits on the Ni-NTA beads was confirmed by high levels of radioactivity measured on the paper filter which captured Ni-NTA beads incubated with $G\alpha_{11}\text{His}$ with no measured $^{35}S$ measurements on the GFC filter (Figure 3.3.4a [right]). Additionally, the specificity of the Ni-his interaction was further confirmed by the approximate 2.7 fold or 3.1 fold higher radioactivity measured on the GFC filter rather than the paper filter when either the $G\alpha_{11}$ or $G\alpha_{11}\text{His}$ proteins, respectively, were assayed in the absence of Ni-NTA beads (Figure 3.3.4b). These results indicate, that while a small amount of $G\alpha_{11}$ subunit may dissociate from the $G\beta\gamma$ dimer upon incubation with GTP$\gamma S$, there is also a substantial percentage of the heterotrimer which remains in the bound state. This result supports a previous study by Leifert et al., the study from which this assay was designed (Leifert et al. 2005b). They also demonstrated that a significant amount of $[^{35}S]\text{-GTP}\gamma S$ bound $G\alpha$ subunits remain attached to the $\beta\gamma$ dimer which is captured by a Ni-NTA bead (Leifert et al. 2005b). In contrast, receptor induced activation resulted in the $[^{35}S]\text{-GTP}\gamma S$ bound $G\alpha$ subunits, separating from the bead-bound $G\beta\gamma$ dimer (Leifert et al. 2005b). This suggests that in the absence of receptor induced activation, the G-protein subunits in solution under these assay conditions, do not completely separate. This is in agreement with the TR-FRET results shown in Figure 3.3.3.

### 3.3.5 Validation of G-protein interaction assays

Results mentioned earlier in this chapter (section 3.1.5) demonstrate that the G-protein

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$^1$ The negligible amount of radioactivity from non-hexahistidine $G\alpha_{11} + G\beta_1\gamma_2$ non-specifically bound to Ni-NTA beads is subtracted to give results of only $G\alpha_{11}$ specifically bound via hexahistidine-tagged $G\beta\gamma$
interaction as measured using TR-FRET, can be hindered in the presence of a competing binding partner (Figure 3.1.7). An excess of either one of the subunits, $G\alpha$ or $G\beta\gamma$, resulted in a significant decrease (approximately 60-80%) of the TR-FRET signal obtained due to heterotrimer formation. This indicated a displacement of the labelled G-protein away from its binding partner. Further investigation into potential binding partners for the G-protein using the TR-FRET assay are described here. These displacement assays were conducted so the tested competitor was introduced to the system once steady-state TR-FRET interaction had been established (usually after a ~5 minute incubation). The fluorescence measurement could be made throughout the competition assay and the displacement of the fluorescently labelled protein could be monitored as can be seen by the decrease in fluorescence in Figure 3.1.7 a) and b). The same decrease was not observed in the presence of BSA or lysozyme, neither of which are known for undergoing a specific interaction with either of the G-protein subunits (appendix 6.1). Specific G-protein interactions or activation is demonstrated in Figure 3.3.5 by the introduction of $G\alpha_i$His or $\text{AlF}_4^-$ respectively (positive controls). In addition, regulator of G-protein signalling 4 (RGS4), which was investigated by Tamara Cooper in my laboratory for its ability to bind to the $G\alpha$ subunit, as well as a peptide known as Tachyplevin (discussed in detail in section 3.3.5.1) were also shown to induce G-protein separation. A peptide sequence not known to specifically interact with either of the G-protein subunits (amino acid sequence: Gly-Ala-Glu-Gly-Cys-Pro-Gly-Cys-Cys-Gly-Gly-Gly), did not show a significant decrease in FRET fluorescence, indicating the subunits did not separate.

![Figure 3.3.5 Heterotrimer dissociation induced by G-protein interactors](image)

**Figure 3.3.5 Heterotrimer dissociation induced by G-protein interactors:** Steady state TR-FRET between 5 nM $G\alpha_i$His Tb and 15 nM $\beta_1\gamma_2$His Alexa significantly decreases in the presence of $G\alpha_i$His; Tachyplesin; RGS4His; and $\text{AlF}_4^-$, but not in the presence of a non-specific peptide. Data represents mean ± SEM; n = 3.
As expected, both the G\(\alpha_i\)His and RGS4 proteins were able to effectively compete for the binding of the labelled G\(\beta\gamma\) or G\(\alpha\), respectively. Regulators of G-protein signalling are GTPase activating proteins (GAP), known for their ability to increase the rate of GTP hydrolysis undertaken by the G\(\alpha\) subunit during the G-protein activation/deactivation cycle. The RGS4 protein has been previously reported to bind to the G\(\alpha_q\) subunit with high affinity, in various states of activation (GDP [inactive]; GTP\(\gamma\)S [active]; and AIF\(_4^–\) [transitional]) (Dowal et al. 2001), as well as the G\(\beta\gamma\) (Dowal et al. 2001). Additionally, structural studies (Tesmer et al. 1997) as well as FRET (Kimple et al. 2003) and the TR-FRET studies conducted in our lab (Leifert et al. 2006) demonstrate the RGS4 interaction with the G\(\alpha_i\) subunit.

No significant decreases in FRET fluorescence occurred in the presence of a peptide with an amino acid sequence which is not reportedly specific for interactions with either of the heterotrimer subunits. Interestingly, the G-protein heterotrimer was shown to separate, or at least the number of fluor molecules within the Forster radius decreased, in the presence of the peptide, tachyplesin.

### 3.3.5.1 Tachyplesin

Tachyplesin is a major granular component of hemocytes (found in the hemolymph of invertebrates), and was first isolated from horseshoe crabs (Nakamura et al. 1988). It is a 17 amino acid antimicrobial peptide with activity against fungi, gram positive and gram-negative bacteria (Nakamura et al. 1988). Tachyplesin has structural properties similar to mastoparan, a cationic peptide from wasp venom. Mastoparan directly interacts with G-proteins, in particular the C-terminus of the G\(\alpha_i\) subunit (Weingarten et al. 1990), and is thought to mimic the effect of ligand activated GPCR on G-proteins. The amino acid sequence of tachyplesin (specifically tachyplesin I, used in this study) is Lys-Trp-Cys-Phe-Arg-Val-Cys-Tyr-Arg-Gly-Ile-Cys-Tyr-Arg-Cys-Arg (Muta et al. 1990), while the amino acid sequence of the mastoparan peptide is Ile-Asn-Leu-Lys-Ala-Lys-Ala-Lys-Asn-Leu-Lys-Ala-Lys-Ala-Lys (Higashijima, Burnier & Ross 1990). Structural similarities between the tachyplesin and mastoparan peptides include a relatively high content of basic amino acids (lysine and arginine), an amphiphilic structure and an amidated carboxyl terminus (Kurata, Ariki & Kawabata 2006).
Mastoparan (Ariki *et al.* 2004) and tachyplesin (Ozaki, Ariki & Kawabata 2005) are both capable of inducing exocytosis of granular hemocytes of horseshoe crabs via a pertussis toxin sensitive, G-protein dependent, signalling pathway (Gαi1 is pertussis toxin sensitive) (Ariki *et al.* 2004). The Gα subunit (particularly from the Gαi/o family), has been shown to directly bind to both mastoparan and tachyplesin using surface plasmon resonance studies, with Kd = 0.22 μM and Kd = 0.88 μM, respectively (Ozaki, Ariki & Kawabata 2005). The TR-FRET result shown in Figure 3.3.5 supports this notion in that tachyplesin was shown to decrease the interaction between Gα1Tb and Gβ1γ2Alexa and resulted in a decrease in the FRET between the subunits.

Fluorescence microscopy methods were employed in order to provide further evidence of the Gα-tachyplesin interaction, independently from the TR-FRET system (Figure 3.3.6).

<table>
<thead>
<tr>
<th>No Protein</th>
<th>Gα1His (2.5μM)</th>
<th>Gβ1γ2His (2.85μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC labelled Tachyplesin (FITC filter)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alexa546 labelled β4γ2 (Cy3 filter)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Figure 3.3.6 Fluorescence microscopy of Gα1His is specific capture of fluorescently labelled interactors*: Ni-NTA beads displaying no protein (far left), Gα1His (middle), or Gβ1γ2His (far right) are incubated with either FITC labelled tachyplesin or Alexa546 labelled β4γ2 and non-bound fluor removed by washing. The brightest fluorescence can be seen on beads displaying Gα1His on the surface. Scale bars in images represent 200 μm.

In this study, Ni-NTA beads were pre-incubated with either the Gα1His or Gβ1γ2His for 1 hour, then washed to remove unbound proteins. These beads were then exposed to either a FITC labelled tachyplesin peptide (top row), or Alexa 546 labelled Gβ4γ2 protein. Beads were washed repeatedly to remove unbound fluorescent moieties and mounted onto a microscope slide with cover slip. Fluorescence was monitored using an Olympus BX 41 fluorescence microscope fitted with a FITC filter (Figure 3.3.6 top row) and a Cy3 filter (Figure 3.3.6 bottom row). Qualitative data from these fluorescence
microscopy studies (Figure 3.3.6) confirmed results shown in the TR-FRET interaction assay, whereby the higher levels of fluorescence, indicating the presence of the labelled binding partner, were only observed on beads displaying the G\(\alpha_{i1}\)His protein. This is the second time tachyplesin has been shown to interact with G-proteins and agrees with the report of Ozaki and coworkers (Ozaki, Ariki & Kawabata 2005). This result further validates the ability of the TR-FRET assay to identify specific G-protein interactors and indicates its potential as a screening system for the ability of various compounds to alter G-protein function. Indeed, the basic premise of the developed assay herein has now been applied to routinely screen pharmaceutical compounds in a University of Michigan biomolecule screening program.

### 3.3.6 Increasing assay throughput

Increasing assay throughput can be achieved by assay miniaturization. Assay miniaturization is dependent on multiple factors, including the need for a homogeneous assay, the ability to handle ultra-low volumes of liquid, and the demand for sensitive readouts (Gribbon & Sewing 2003). It has previously been reported that fluorescence polarization assays monitoring either estrogen receptor \(\alpha\)/ligand, or protein kinase/serine containing peptide substrate interactions, were capable of adaptation to a 384 or 1536-well format (100 \(\mu\)l to 2.5 \(\mu\)l volumes) with minimal changes to the system (Kowski & Wu 2000). Figure 3.3.7 demonstrates the adaptability of the TR-FRET G-protein interaction assay to show a > 2.5 fold increase in a 10 \(\mu\)l assay volume in a 384-well black plate.
Optimal conditions required attention to the geometry of the microtiter plate and the detection parameters of the plate reader (Victor\textsuperscript{3}).

### 3.3.7 TR-FRET reconstitution assays

The G-protein interaction TR-FRET assay (described in section 3.1.5) provides a seemingly ideal platform for the development of a TR-FRET assay capable of monitoring receptor induced activation of the G-proteins. In this way, simply by monitoring the changes in fluorescence as the distance between the fluors on the heterotrimer change during activation, the G-proteins can act as a molecular switch, indicating when the receptor has been turned on or off. The assays were conducted with appropriately labelled G-proteins (as used in the above sections), GPCR membrane preparation, GTP\textgamma\textsubscript{S} and ligands at concentrations similar to those used in the [\textsuperscript{35}S]-GTP\textgamma\textsubscript{S} assay (see section 2.3.4). Assays were originally monitored after an incubation time of 90 minutes in order to see an accumulated effect of separated G-proteins induced following receptor stimulation. This accumulation effect was expected since GTP\textgamma\textsubscript{S}-bound G\textalpha was not expected to re-unite with the G\beta\gamma subunit. This idea is supported by reports that the separation of the G-proteins in the presence of [\textsuperscript{35}S]-GTP\textgamma\textsubscript{S} is initiated by activated receptor in vitro (Leifert \textit{et al.} 2005b). However, results indicated little change in TR-FRET (or net FRET) in the presence of agonist, or agonist + antagonist. It also became apparent that the system, regardless of the time-resolved
techniques, was vulnerable to interferences of coloured compounds introduced into the system. This was particularly evident upon the addition of the $\alpha_{2a}$-adrenergic receptor antagonist UK-14304 which is yellow in colour.

Initial TR-FRET experiments used the receptor membrane extract, in the same form used in the $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ assays. These results indicated that the membrane extract may prove problematic, as effects such as decreases in TR-FRET emission over time, were observed.

![Figure 3.3.8](image1)

Figure 3.3.8 TR-FRET reconstitution assay of $\alpha_{2a}$-Adrenergic receptor with varying concentrations of GTP$^\gamma$S. Net FRET of a reconstitution mix consisting of 10 nM $\beta_{y}\text{Tb} + 10$ nM G$\alpha_{i1}\text{His Alexa}; 0.1$ mg/ml $\alpha_{2a}$-adrenergic receptor. (a) No GTP$^\gamma$S; (b) 1 $\mu$M GTP$^\gamma$S; (c) 100 $\mu$M GTP$^\gamma$S. Assays were conducted in 100$\mu$l TMND in the presence (○) or absence (▼) of 10 $\mu$M adrenaline. Excitation = 340 nm; Emission = 545 nm and 572 nm with a 100 $\mu$s delay and 900 $\mu$s count time.

As seen in Figure 3.3.8, the TR-FRET measurement (as measured by 572 nm emission/545 nm emission), decreases over time, regardless of the presence of receptor agonist. At the point of $t = 90$ minutes, the point at which the assay was previously being read, the signals are indistinguishable from one another. However at earlier time points in the reaction, there is a difference in the Net FRET measured in the presence of activated receptor (epinephrine), which is only apparent in the presence of GTP$^\gamma$S. However, this difference between the presence and absence of the cognate agonist is not statistically significant, but a clear trend can be observed.
The correct microenvironment of the receptor is essential for retention of integrity. However, the crude membrane preparation was proving problematic due to its intrinsic effect on the fluorescence output over time. While assay interferences are unlikely to be arising from autofluorescence or scattering of the incident light due to the time gated emission (as discussed in section 3.1.4.1), contributing artefacts to the signal may arise from inner filter effects arising from the turbidity of the membrane preparation used in these assays. The inner filter effect, in this case could be used to describe a decrease in light transmission though the assay solution by light absorption of the incident or emitted light or scattering of the emitted light. It has previously been reported that the level of turbidity of a sample is related to the particle size within the sample (Pozharski, McWilliams & MacDonald 2001), and for this reason, the membrane extract was extruded sequentially through polycarbonate filters (Whatman track-etch) with pore diameters of 1.0 μm, then again through the filters with 400 nm pore diameters to produce smaller extract particle sizes and therefore a less turbid sample for use in the TR-FRET assay. The solution became clearer once it had been extruded (for image of turbidity change see appendix 6.2), and receptors remained functional (refer to section 4.3.6.1 in the following chapter). Additionally, the TR-FRET signal remained more stable over time (Figure 3.3.9).

![Figure 3.3.9 Decrease in TR-FRET emission in the presence of extruded membrane vesicles containing M2-muscarinic receptor in the presence of cognate agonist, carbachol](image)

Extruded membrane preparations containing vesicles of diameter <400 nm were used in the TR-FRET reconstitution assay (0.05 mg/ml) with 20 nM Gαi1His Alexa; 20 nM β1γ2His Tb; 50 nM GTPγS in 100 μl TMN. Carbachol (120 mM) dependent decrease in fluorescence emission at 572 nm was observed when using vesicles composed of M2-muscarinic receptor containing membranes. This increase in fluorescence was no longer observed in the presence of antagonist, atropine (100 μM). The same fluorescence changes were not observed for vesicles composed of cell membranes not containing the M2-muscarinic receptor (NI - non-infected cells). (a) M2R vesicles incubated with buffer (basal, red [■]); 120 mM carbachol (blue [▲]); 120 mM carbachol + 100 μM atropine (black [▼]); (b) Non-infected liposomes incubated with buffer (basal, red [■]); 120 mM carbachol (blue [▲]); 120 mM carbachol + 100 μM atropine (black [▼]). n = 2.

Results shown here indicate a decrease in TR-FRET over time as carbachol (an M2R
agonist) is present in the assay mix (Figure 3.3.9a). This phenomenon was only observed when the muscarinic receptor was present in the assay (Figure 3.3.9a) and non-infected membrane extracts (extruded) in the same assay did not show any changes in net FRET measurements when carbachol was introduced (Figure 3.3.9b). Additionally, a decrease in net-FRET was not observed when 100 μM atropine (M2R antagonist) was introduced into the assay simultaneously with 120 mM carbachol (agonist). However, the ratio made up of the 572 nm data (from the Alexa emission) over the 545 nm data (from the terbium) may be deceiving. The resulting decrease in 572 nm/545 nm data is due primarily to the increase in 545 nm signal from the emission peak of the terbium. Normally, this ratio is plotted to show that the decrease in FRET emission of the acceptor fluorophore is accompanied by an increase in fluorescence emission of the donor fluorophore. This is because the energy from the donor used during the resonance energy transfer process when in close proximity to the acceptor, now escapes as fluorescence of the donor probe. While data here shows an increase in donor probe emission, it is unclear as to whether this is due to a separation of the FRET pair, due to the fact that a corresponding decrease in acceptor emission is not observed. Some of the shortcomings of organic fluorophores in fluorescence assays include the sensitivity to environmental changes and the vulnerability to quenching from other components within the assay system. It may be due to this latter reason that clear changes in the 572 nm data cannot be measured during the receptor/G-protein reconstitution TR-FRET assay as they were in the G-protein TR-FRET interaction assay described in previous sections in this chapter. However, the terbium emission, however is less sensitive and therefore monitoring the changes in fluorescence at 545 nm may be an indication that the fluorophores are separating (and hence donor emission increases), but the subsequent increase in acceptor emission was not observed. Alternatively, the increase in terbium emission could be resulting in a large increase in the background emission of terbium bleeding through at the emission wavelength of Alexa546 (572 nm) (evidence of terbium background at 572 nm can be seen in Figure 3.1.6). This increase in background terbium emission may be masking any changes in emission resulting from the Alexa fluorophore.
3.4 Chapter Summary

The TR-FRET assay using functional G-proteins labelled with small organic fluors, was validated for its ability to monitor specific G-protein interactions. Assay throughput can be increased in well-based format to 384-well plates and the volume of the assay can be reduced to as low as 10 μl. This TR-FRET assay with G-proteins could be used to discover interactors/modulators of G-protein signalling acting directly at the G-proteins, such as Tachyplesin and RGS4. This is of interest, as it can provide a mode of discovering therapeutic interventions independent of the receptor by targeting the G-proteins themselves (Holler, Freissmuth & Nanoff 1999). Results also indicated that the G-protein heterotrimer TR-FRET assay can be coupled to a G-protein coupled receptor to monitor ligand induced activation, although further investigation and refinement is still required. This assay platform, due to its homogeneous design, could potentially be adaptable to a surface bound format for array or sensor applications. Surface capture of the signalling assembly is further investigated in the next chapter (chapter 4).