Probing the Oligomeric Status of G Protein-Coupled Receptors by Förster Resonance Energy Transfer and Single-Particle Fluorescence

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Pharmaceutical Sciences
University of Toronto

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Abstract

Much evidence indicates that G protein-coupled receptors can form oligomers, but the size and stability of those oligomers has not been well characterised. We used single-particle tracking (SPT) and Förster resonance energy transfer (FRET) to measure the oligomeric size of the M2 muscarinic receptor, a prototypical class A GPCR, in live cells. Single-particle intensity distributions that we obtained for the monomeric control CD86 and the dimeric control CD28 are nearly identical, and no conclusion about the oligomeric size of M2 receptors could be drawn from SPT measurements. FRET measurements allowed us to distinguish the monomeric control CD86 from the dimeric controls CD28 and caveolin-1, and the pattern of efficiencies produced by M2 receptors is similar to the pattern produced by the monomers but not the dimers. The view of M2 muscarinic receptors as transient oligomers appears to be the most consistent with other studies using different biochemical and biophysical techniques.
Acknowledgments

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List of Abbreviations

Cav-1: Caveolin-1, a membrane scaffolding protein

CD28: Cluster of differentiation 28 receptor (disulphide-linked dimer).

CD86: Cluster of differentiation 86 transmembrane protein (monomer, also known as B7.2)

CHO cells: Chinese hamster ovary cells

CUDA: Compute Unified Device Architecture (parallel computing platform and programming model for NVIDIA graphics cards)

FLIM: Fluorescent lifetime microscopy

FRAP: Fluorescence recovery after photobleaching

FRET: Förster (fluorescence) resonance energy transfer

GPCR: G protein-coupled receptor

HEK293 cells: Human embryonic kidney cells

M2: M2 muscarinic receptor, a prototypical class A GPCR

M2R1: M2 muscarinic receptor fused to the coiled coil domain from the GABA_B1 receptor

M2R2: M2 muscarinic receptor fused to the coiled coil domain from the GABA_B2 receptor

M2C457A: M2 muscarinic receptor with the palmitoylated cysteine at position 457 mutated to an alanine

MP: Myristoylation palmitoylation tag derived from the first 32 amino acids of the human Gαi1 protein

PALM: Photoactivated localisation microscopy

SPT: Single-particle tracking

TIRF: Total internal reflection fluorescence
Chapter 1
Introduction
1 Introduction

1.1 G protein coupled receptors

G protein coupled receptors (GPCR) are a class of cell surface receptors that span the membrane seven times and signal by activating heterotrimeric G proteins. Humans express over 800 different GPCRs that can respond to a wide range of agonists including amino acids, peptides, proteins, lipids, biogenic amines, ions, odorants and light [1]. About 30 GPCRs are targeted by approximately 27% of all FDA-approved drugs [2], while over 100 orphan receptors do not have a known agonist or physiological role [3,4].

Mutated GPCRs are responsible for a large number of genetic disorders [5], and dysfunctional GPCR signalling has been implicated in many psychiatric illnesses [6,7] and cancers [8-10]. For example, constitutively active mutants (CAMs) of rhodopsin, which signal even in the absence of an agonist, are responsible for night blindness and retinitis pigmentosa. Inactivating missense and nonsense mutations of the calcium-sensing receptor are responsible for familial hypocalciuric hypercalcemia, while activating mutations are responsible for autosomal dominant hypocalcemia, autosomal dominant hypoparathyroidism and hypocalcemic hypercalciuria. Several polymorphisms and loss-of-function mutations of the melanocortin 4 receptor can cause binge eating and obesity. Single nucleotide polymorphisms, insertions and deletions of the vasopressin 2 receptor can cause familial nephrogenic diabetes insipidus [5]. Some of those disorders are caused by the retention of the mutated receptor in the endoplasmic reticulum, and the phenotype sometimes can be rescued by using cell-permeable receptor ligands that stabilise the disordered receptor and improve its trafficking to the cell surface, in a process called “pharmacological chaperoning” [8,11,12].

GPCRs can be subdivided into five different families based on sequence homology. The GRAFS acronym, which stands for Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin, can be used to remember the most notable members of each family [9]. Families containing rhodopsin-like, secretin-like and glutamate-like receptors can also be referred to as class A, class B and class C.
GPCRs, respectively [13]. Type 1 vomeronasal receptors and type 2 taste receptors share very little sequence homology with all other GPCRs, and recent classifications have included those receptors as two additional families [14,15].

1.2 GPCR-mediated signalling

The traditional view of GPCR signalling is shown in the schematic in Figure 1.1. In brief, an agonist binds to a GPCR and enhances its ability to activate G proteins. The activated GPCR acts as a guanine nucleotide exchange factor and stabilises the open conformation of the heterotrimeric G protein, which allows GDP to dissociate from and GTP to associate with the alpha subunit. Once the alpha subunit is bound to GTP, the receptor-G protein complex falls apart and the Gα and Gβγ subunits of the heterotrimeric G protein go on to activate or inhibit separate downstream effectors. Eventually the Gα subunit hydrolyses GTP to GDP, through its intrinsic GTPase activity. The rate of hydrolysis can be increased through the action of GTPase activating proteins (GAPs). The GDP-bound Gα subunit can then re-associate with the Gβγ subunit, and the heterotrimeric G protein can once again interact with a GPCR and repeat the cycle.

Many aspects of GPCR signalling still are under investigation. For instance, it is debated whether receptors and G proteins couple transiently or if they form stable complexes [16]. GPCRs can be coimmunoprecipitated with G proteins [17], which suggests that the interaction between a GPCR and a G protein is stable. However, there is amplification of the signal at the GPCR-G protein level [18], and Gαs and Gα2 have been reported to move into the cytosol within seconds or minutes of receptor activation [19,20], which suggests that each GPCR can activate multiple G proteins and that the interaction is transient [21]. Activated receptors stabilise the open conformation of the Gα subunit, which allows GDP to leave and GTP to enter the nucleotide-binding pocket [22]. In the absence of GDP or GTP, activated receptors and nucleotide-free G proteins form high-affinity complexes, which are sparse in live cells due to abundance of GTP but become the dominant species in *in vitro* experiments conducted at a subsaturating
concentration of the nucleotide [23]. Thus, many in vitro experiments may overestimate the stability of the interaction between receptors and G proteins, as they are actually measuring the stability of high-affinity complexes formed between receptors and nucleotide-free G proteins [22,24].

G proteins can be activated without GPCRs by guanine nucleotide exchange factors (GEFs) and activator of GPCR signalling (AGS) proteins, and GPCRs can activate effectors without G proteins, either through β-arrestin-mediated pathways [25] or by interacting directly with receptor and non-receptor tyrosine kinases (e.g. src) [26,27] and other proteins [28]. Furthermore, GPCRs, G proteins and other components of the signalling cascade may cluster in specific microdomains on the cell membrane, which increases the specificity and the rate of signalling [29-32].
Figure 1.1: A schematic showing the traditional view of class A GPCR signalling. Class A GPCRs have a wide range of ligands, including biogenic amines, amino acids, ions, lipids, peptides and photons. Once a GPCR is bound to an agonist, it can facilitate the exchange of GDP for GTP in the heterotrimeric G protein, leading to the dissociation of the Ga and Gβγ subunits. The Ga and Gβγ subunits go on to activate or inhibit separate downstream effectors, which increase or decrease the production of second messenger molecules and may affect the expression of different genes. This schematic was adapted from Dorsam and Gutkind [10].
1.3 GPCR oligomers

Perhaps the biggest amendment to the original model of GPCR signalling comes from the accumulating amount of evidence suggesting that GPCRs exist and function as oligomers. The evidence is clearest for class C GPCRs, which have been shown to form stable dimers. For example, the GABA_B1 and GABA_B2 receptors exist as heterodimers that are stabilized by the coiled-coil domains present on their C-termini [33]. The heterodimer is functionally important because GABA_B2 receptors cannot bind agonists and are functional only as heterodimers with GABA_B1, and GABA_B1 receptors are expressed on the cell membrane only if their endoplasmic reticulum retention sequence is masked through receptor heterooligomerisation. In contrast to the GABA receptors, other class C GPCRs, such as the calcium receptors, metabotropic glutamate receptors (mGluRs) and taste-1 receptors, lack the coiled-coil domains and are stabilised by disulphide bonds between their extra-cellular Venus flytrap motifs [34-36]. Mutations that prevent the formation of those disulphide bonds also disrupt receptor dimerisation, which typically increases the affinity of the receptors for their agonist [5]. In fact, many gain-of-function mutations of the calcium receptor cluster around or involve cysteines 129 and 131, which are responsible for the formation of the inter-receptor disulphide bond [37]. While there is evidence that class C GPCR dimers may come together into tetramers or higher-order oligomers, the stability and the functional importance of those oligomers still is a matter of debate [33,38,39].

Many attempts have been made to measure the oligomeric size of class A GPCRs. However, the results obtained by different groups often are contradictory, and it still is not clear whether class A receptors associate into oligomers. Since no coiled-coil associations or disulphide bonds are formed between class A GPCRs, oligomers formed by those receptors are likely to be less stable than those formed by class C GPCRs [40]. This could be one of the reasons why the oligomeric size of class A receptors has been more difficult to quantify.

Rhodopsin was the first class A GPCR to be cloned and characterised. Initial studies on rhodopsin, using transient flash-induced photo-dichroism, flash-bleaching recovery, X-ray and neutron diffraction and electron microscopy, all have indicated that rhodopsin exists as a
randomly-distributed monomer in native rods [41]. However, a more recent study that used atomic force microscopy to image native disk membranes, produced images of rhodopsins arranged in pseudo-crystalline arrays of dimers or higher-order oligomers [42]. Different biochemical and hydrodynamic methods, such as frictional ratio measurements, gel filtration chromatography, crosslinking, sedimentation velocity analysis and small-angle neutron scattering, all showed that rhodopsin exists as a monomer when native rod membranes are solubilised in most detergents (e.g. Triton X-100, DDAO, Cemulsol LA90 and dodecanoyl octaoxyethylene glycol) [43]. However, when rhodopsin is solubilised in cholate or low concentrations of DDM, it can form oligomers of various sizes, and flash-activation of rhodopsin can induce oligomerisation, flocculation and precipitation in many detergents [43].

Results obtained for other class A GPCRs are just as inconsistent. For example, Flag-, Myc- and HA-tagged M2 muscarinic receptors expressed together in Sf9 cells were sequentially coimmunoprecipitated using antibodies against two of the three epitopes, and immunoblotted using antibodies against the third epitope, which suggests that at least some of the receptors form trimers or higher-order oligomers [44]. However a separate study found that the serotonin 5-HT1A receptor can be coimmunoprecipitated with a wide range of different GPCRs, including 5-HT1B, 5-HT1D, EDG1, EDG3, GPR26 and GABA2 receptors [45]. This suggests either that most GPCRs have a propensity to oligomerise, or that coimmunoprecipitation is not a very specific technique for detecting protein-protein interactions between membrane proteins, especially if those proteins are over-expressed in transiently transfected cells [46].

Radioligand binding experiments using different class A GPCRs reveal multiple classes of sites even in the absence of G proteins, which suggests that at least some of the receptors must form oligomers [47]. However, studies performed on receptors isolated in lipid nanodisks have shown that a single β2 adrenergic receptor can activate its cognate G protein [48], and a single rhodopsin can activate transducin, can be phosphorylated by GRK1 and can bind arrestin-1 with a high affinity [49-51]. This suggests that the monomeric receptor is the minimal functional unit.

Recently-developed biophysical techniques, such as single-particle tracking (SPT) and Förster resonance energy transfer (FRET), have made it possible to examine the oligomeric size of
membrane proteins in live cells. However, the results obtained using those techniques have been just as inconclusive as the results obtained using the more traditional biochemical and pharmacological approaches. For example, while single-particle tracking has been used to show that the M1 muscarinic receptor and the N-formyl peptide receptor exist as a mixture of monomers and dimers in live cells [52,53], FRET has been used to show that many other class A GPCRs exist exclusively as dimers or higher-order oligomers [54-60].

1.4 Outline of the thesis
The goal of this project was to measure the stability and the relative proportions of monomers, dimers and higher-order oligomers of the M2 muscarinic receptor, a prototypical class A GPCR, in live cells. Chapter 2 describes our attempt to reach this goal using single-particle tracking. Chapter 3 describes our attempt to reach this goal using cell-averaged FRET measurements. Both techniques come with a different set of challenges that have not been fully addressed in previous studies.
Chapter 2
Single-particle tracking
2 Single-particle tracking

2.1 Abstract

In two recent studies, the authors tracked fluorophore-labelled molecules of the M<sub>1</sub> muscarinic receptor and the N-formyl peptide receptor, and concluded that both receptors exist as a 70:30 mixture of monomers and dimers in live cells. We used a similar single-particle tracking approach to measure the oligomeric size of the M<sub>2</sub> muscarinic receptor, which has been reported to exist as a tetramer. The integral membrane proteins CD86 and CD28 were used as monomeric and dimeric controls, respectively, and each construct was fused with a SNAP tag at its N-terminus and was labelled with SNAP-Surface 488. Chinese hamster ovary cells expressing each construct were imaged on a total internal reflection fluorescence (TIRF) microscope to obtain time-lapse movies of labelled proteins moving along the cell membrane. Those movies were analysed using publicly available software to obtain a list of intensities and trajectories for all molecules in the movie. Three different algorithms were used to detect and measure the intensities of single molecules, but in each case the resulting distributions of intensities showed single asymmetric peaks that could be fit well by a log-logistic distribution. The main factors determining the mean and standard deviation of the distributions were the level of expression and the average intensity of the images, and cells expressing monomeric CD86, dimeric CD28 and the M<sub>2</sub> receptor at a comparable level showed single-molecule intensity distributions that were nearly the same. These and other results illustrate the difficulty in using single-particle tracking to measure the oligomeric size of transmembrane proteins.
2.2 Introduction

The basic premise of single-particle tracking (SPT) techniques is simple: a fluorescently labelled membrane protein is imaged using a total internal reflection fluorescence (TIRF) microscope, single fluorescent particles are detected and their fluorescence intensity is recorded as a function of time. Two fluorophores should be twice as bright as one, and so a dimer presumably would show twice the fluorescent intensity of a monomer and would show two photobleaching steps instead of one. The same principle should also apply to higher order oligomers.

Over the last three years, several groups have used SPT to measure the oligomeric size of different class A GPCRs. Hern et al. used fluorophore-tagged M₁ muscarinic receptor antagonist telenzepine to label M₁ receptors on the plasma surface of live CHO cells [53]. By recording single-particle intensity distributions, counting the number of photobleaching steps and labelling the receptors with two different fluorophores and measuring the degree of colocalisation, the authors showed that M₁ muscarinic receptors exist as a mixture of 70% monomers and 30% dimers. In another study, Kasai et al. used SPT to show that the N-formyl peptide receptor also exists as a mixture of monomers and dimers, with an equilibrium dissociation constant of 3.6 copies/µm² and an average dimer lifetime of 91 milliseconds [52]. Since typical expression levels for GPCRs are below 3 molecules/µm² [61], the results obtained by Kasai et al. suggest a preponderance of monomers at physiological levels of expression.

We used TIRF microscopy and single-particle tracking to try to measure the oligomeric size of the M₂ muscarinic receptor, with CD86 and CD28 used as monomeric and dimeric controls, respectively. All of the constructs were fused with a SNAP tag at the N–terminus, and the proteins were labelled with a cell-impermeable benzyl-guanine derivative dye (i.e., SNAP Surface-488) prior to imaging (Figure 2.3). We obtained time-lapse movies of labelled proteins moving along the basal membrane of the cell, and we used three different algorithms to obtain a list of intensities and trajectories of all the molecules in the movie.
All three proteins showed similar skewed single-particle intensity distributions that are described well by the log-logistic function but are difficult to interpret. Furthermore, random fluctuations in single-particle intensities over time made it difficult to count accurately the number of photobleaching transitions for most detected particles. Thus, we were unable to reach a reliable conclusion regarding the oligomeric size of the M$_2$ receptor from our SPT data.
Figure 2.1: A schematic showing all proteins that were used in the single-particle tracking study. CD86 is an integral membrane protein that is known to be a monomer, and it was used as the monomeric control. CD28 is an integral membrane protein that is known to form disulphide-linked dimers, and it was used as a dimeric control. The M2 muscarinic receptor is a prototypical class A GPCR, and its oligomeric size was being investigated. All three proteins were fused with a SNAP-tag at the N-terminus, which was located at the extracellular side of the membrane in each case. Prior to imaging, cells expressing each protein were exposed to a SNAP Surface dye, and the SNAP-tags catalysed the reaction that led to the covalent attachment of the dye to the protein.
2.3 Materials and methods

2.3.1 Constructs and vectors

Constructs containing the human M₂ receptor were described previously [60], and pcDNA3.1 vectors containing human CD28 and CD86 were a gift from Dr. Bünemann [62]. In order to generate pSNAPf vectors containing CD28, CD86 or the M₂ receptor with a SNAP-tag at the N-terminus, sequences encoding those proteins were amplified through PCR using primers 2F and 2R, 3F and 3R, or 4F and 4R (Table 1), respectively, and were inserted into the pSNAPf vector following the α7 signal sequence and the SNAP-tag sequence, using XhoI and BspEI restriction sites. The α7 signal sequence is a cleavable endoplasmic reticulum targeting sequence, which has been shown previously to improve membrane localization of transmembrane proteins [63]. All sequences were confirmed by DNA sequencing.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2F</td>
<td>CCCCCTCGAGATGCTTGTAGCGTACGACAATG</td>
</tr>
<tr>
<td>2R</td>
<td>GAATTCGCGGCCGCTTATCCGGACCTGCTCCTCTTACTCCTCACC</td>
</tr>
<tr>
<td>3F</td>
<td>CCCCCTCGAGAATGAGACTGCAGACCTGCC</td>
</tr>
<tr>
<td>3R</td>
<td>GAATTCGCGGCCGCTTATCCGGAGCGAGGCCGCTTCTTCTT</td>
</tr>
<tr>
<td>4F</td>
<td>GCCCCTCGAGAATTAACACTCAACAAACTCCTCTAACAATAGCC</td>
</tr>
<tr>
<td>4R</td>
<td>GAATTCGCGGCCGCTTATCCGGACCTTGTAGCGCCTATGTTCTT</td>
</tr>
</tbody>
</table>

Table 1: List of all the primers that were used to generate DNA constructs for the single-particle tracking study.
2.3.2  Cell culture

Chinese hamster ovary (CHO) cells were grown in DMEM media supplemented with 5% fetal bovine serum, 0.5% nonessential amino acids and 0.5% w/w Penicillin-Streptomycin (Invitrogen). Cells were grown at 37 °C in a humidified atmosphere with 5% CO2. For imaging, cells were seeded on number 1 coverslips 10 mm in diameter (Fisher Scientific) inside of six-well plates at a density of 1x10^6 cells per well. Cells were transfected using polyethylenimine (Polyscience Inc.), and were incubated in phenol red- and FBS-free media for 12 to 16 hours prior to imaging.

2.3.3  Imaging

Cells expressing CD28, CD86 or the M2 muscarinic receptor, fused with a SNAP-tag at the extracellular N terminus, were labelled with SNAP Surface-488 or SNAP Surface-549 (New England Biolabs) following the manufacturer’s protocol and were imaged on an Olympus IX81 cellTIRF microscope with a Hamamatsu C9100-13 camera at the SickKids Imaging Facility in Toronto. Image stacks containing 500 to 2000 images were acquired using the Olympus 150x 1.45 N.A. TIRF objective, 1x analog gain, 230x EM gain, and 33 ms exposure time.

2.3.4  Data analysis

Raw image intensities were converted into photon counts using the equation supplied by Hamamatsu (Equation 1). For the C9100-13 camera, \( C_{\text{factor}} = 5.8 \), \( Gain_{\text{analog}} = 1 \), \( Gain_{\text{EM}} = 230 \), \( QE = 90 \). Dark intensity \( N_{\text{dark}} \) was measured by taking an image without laser illumination, and was 2200.

\[
N_{\text{photons}} = C_{\text{factor}} \cdot \frac{\left(N_{\text{raw}} - N_{\text{dark}}\right)}{(Gain_{\text{analog}} \cdot Gain_{\text{EM}} \cdot QE / 100)}
\]
Image stacks were analysed either using customised MATLAB routines (R2012a, The MathWorks, Natick, Massachusetts) or using GMimPro [64].

Single-particle intensity histograms were fit to different models by using the raw intensity data and maximising the corresponding likelihood functions. The arithmetic mean and arithmetic standard deviation of the normal distribution ($\mu$ and $\sigma$ in Equation 2) and the geometric mean and geometric standard deviation of the lognormal distribution ($\exp(\mu)$ and $\exp(\sigma)$ in Equation 3) were obtained analytically, by calculating the mean and standard deviation of the raw data or the natural logarithm of the raw data, respectively. The maximum likelihood fits for the sum of two constrained Gaussians (Equation 4) and the log-logistic distribution (Equation 5) were calculated numerically by using the mle solver in the Matlab statistics toolbox to maximise the fit of the data to the corresponding likelihood functions. In the sum of two constrained Gaussians model, the mean and variance of the second Gaussian were constrained to be twice the mean ($\mu$) and variance ($\sigma^2$) of the first Gaussian, as it is assumed that the second Gaussian arises from the first Gaussian being sampled twice.

\[
F(x; \mu, \sigma) = \frac{1}{\sigma \sqrt{2\pi}} \exp\left(\frac{-(x - \mu)^2}{2\sigma^2}\right)
\]

\[
F(x; \mu, \sigma) = \frac{1}{x} \frac{1}{\sigma \sqrt{2\pi}} \exp\left(\frac{-(\ln(x) - \mu)^2}{2\sigma^2}\right)
\]

\[
F(x; \nu, \mu, \sigma) = \frac{\nu}{\sigma \sqrt{2\pi}} \exp\left(\frac{-(x - \mu)^2}{2\sigma^2}\right) + \frac{(1 - \nu)}{2\sigma \sqrt{\pi}} \exp\left(\frac{-(x - 2\mu)^2}{4\sigma^2}\right)
\]

\[
F(x; \mu, \sigma) = \frac{1}{x} \frac{1}{\sigma \sqrt{2\pi}} \exp\left(\frac{\ln(x) - \mu}{\sigma}\right)
\]

\[
F(x; \mu, \sigma) = \frac{1}{x} \frac{1}{\sigma \sqrt{2\pi}} \exp\left(\frac{\ln(x) - \mu}{\sigma}\right)^2
\]
2.4 Results

2.4.1 Optimising SNAP-tag labelling with benzylguanine-derivative dyes

The TIRF microscope that we used for single-particle tracking experiments was equipped only with the 491 nm and 561 nm laser lines, which limited our choice of fluorophores from the NEB catalogue to SNAP-Surface 488, SNAP-Surface Alexa Fluor 488, SNAP-Surface 549 and SNAP-Surface Alexa Fluor 546. CHO cells produced more autofluorescence when excited with the 491 nm laser than the 561 nm laser, probably due to the presence of flavins and flavoproteins that absorb strongly at 488 nm [65]. Thus, the first fluorophore that we used was SNAP-Surface 549, which could be excited strongly with the 549 nm laser. However, when we imaged cells expressing proteins labelled with SNAP-Surface 549, we detected high levels of non-specific fluorescence coming from the cells and the coverslip, and even untransfected cells labelled with SNAP-Surface 549 gave a strong fluorescent signal (Figure 2.2, panels A to C). Thus, SNAP-Surface 549 could not be used for single-particle tracking experiments. The next fluorophore we used was SNAP-Surface 488. The overall signal-to-noise ratio was lower for SNAP-Surface 488 than for SNAP-Surface 549, but the amount of non-specific adsorption to the cells and the coverslip also was distinctly reduced (Figure 2.2, panels D to F). Thus SNAP Surface-488 was used throughout the rest of this study.

We did not measure the efficiency of labelling of SNAP-tagged proteins with SNAP Surface-549 and SNAP Surface-488. However, the manufacturer’s protocol and previous studies that used the SNAP-tag technology [38] report that near-complete labelling is achieved.
Figure 2.2: TIRF images of untransfected CHO cells labelled with SNAP-Surface dyes. Panels A to C show cells that were labelled with Snap-Surface 549 and were excited using the 561 nm laser. Panels D to F show cells that were labelled with SNAP-Surface 488 and were excited using the 491 nm laser.
2.4.2 Optimising the image analysis pipeline

Many software packages exist for the detection and tracking of single particles in TIRF images, and each package offers distinct advantages and disadvantages in terms of the accuracy of the algorithms that are employed and the speed with which the data are analysed. In order to find the best available software for the analysis of our data, we compared three different packages—GMimPro, u-track and GPU-fit—on a subset of our data from cells expressing the monomeric control CD86.

GMimPro [64] detects single particles by sliding a 7 x 7 pixel Gaussian mask over the image and measuring the amount of correlation between the mask and the image. If the correlation exceeds a certain threshold at a given location in the image, that location is recorded as a single particle. The amount by which the Gaussian mask has to be scaled in order to best-reproduce that section of the image corresponds to the intensity that is recorded for that particle. In order to track single particles from one image to the next, GMimPro uses a closest approach algorithm, which links two spots in adjacent frames that are closest to each other and ends a trajectory if it does not find a spot in the subsequent frame within a predefined distance. The main advantage of GMimPro is that it can detect and track single molecules very quickly, as it employs relatively simple algorithms and is distributed as precompiled C++ code.

U-track [66] detects single particles by assembling a list of pixels whose intensity deviates significantly from the average intensity of the image, and fitting one or more Gaussians to the region around each of those pixels, using a least-squares fit. If a good fit is achieved, candidate spots are accepted as single particles, with an intensity given by the area of the corresponding Gaussian. The optimal number of Gaussians is determined using the Bayes information criterion. In order to track single particles, u-track first links particles that are in close proximity in adjacent frames and then joins those tracks to close gaps and try to capture merging and splitting behaviour.

GPU-fit [67] is an algorithm that can fit cropped regions of an image to a two-dimensional Gaussian with Poisson noise (Figure 2.5) and can achieve very high throughput if it is run on a
CUDA-compatible graphics card. It was implemented as an alternative to the least-squares Gaussian fit in u-track, which incorrectly assumes Gaussian noise and is prohibitively slow when analysing long image stacks. One disadvantage of GPU-fit is that it can fit each spot only to a single Gaussian, potentially making it less robust than u-track at high levels of expression. The detection of candidate spots and the linking of single particles still were performed using u-track, and the fitting algorithm was the only part that was changed.

In order to compare the performance of GMimPro, u-track and u-track with GPU-fit on the same set of data, we obtained 1000 timelapse images of CD86 molecules, fused with a SNAP-tag at the extracellular N-terminus and labelled with SNAP-Surface 488, moving on the basal surface of live CHO cells. Panels A and B in Figure 2.3 show an image of a cell before photobleaching, and panels C and D show an image of the same cell after 30 seconds of photobleaching, which was done by continuously imaging the cell for 30 seconds. Panels A and C show the raw data while panels B and D show background-corrected images using an artificial colouring scheme. Green circles in B and E surround all single-particle spots that were detected using u-track with GPU-fit. An example of a two-dimensional fit that is achieved by GPU-fit for one of those spots is presented in Figure 2.5. Spots that were detected on the edges of the cell or outside the cell were removed during post-processing and were not included in the single-particle intensity histograms or the photobleaching-step analysis.

When the first 20 frames of the time-lapse movie of a cell shown in Figure 2.3 were analysed using GMimPro, u-track, or u-track with GPU-fit, the obtained single-particle intensity histograms always were asymmetrical and were skewed to the right (Figure 2.4, panels A, B and C, respectively). The distributions could not be described by a single Gaussian (Figure 2.4, cyan lines) and was described better by a log-logistic distribution (Equation 5; Figure 2.4, red lines) than a sum of two constrained Gaussians (Equation 4; Figure 2.4, green lines). The log-logistic distribution was used instead of the log-normal distribution because while both distributions have a similar shape, the log-logistic distribution would typically produce a better fit with the same number of parameters. Single-particle intensity distributions obtained from 20 consecutive images acquired after 30 seconds of photobleaching are shown in panels D, E and F in Figure
2.4. The log-logistic distribution (Equation 5; Figure 2.4, red lines) still fit the data better than the sum of two constrained Gaussians (Equation 4; Figure 2.4, green lines) or a single Gaussian (Figure 2.4, cyan lines). The sum of two Gaussians model has been used previously to extract the relative proportions of monomers and dimers from single-particle intensity distributions [52], but the key assumption of this model is that the first Gaussian corresponds to the single-particle intensity of the monomer and the second Gaussian corresponds to the single-particle intensity of the dimer. If this assumption was correct, the single-particle intensity distributions would lose the heavy tail and become progressively more Gaussian as the molecules are photobleached. Instead we observe that the shapes of the distributions are almost identical before and after photobleaching, and this suggests that using a sum of two or more Gaussians is not an appropriate way to model or analyse our data. The log-logistic distribution achieves a surprisingly good fit to the data acquired both before and after photobleaching, especially when the data are analysed using u-track with GPU-fit (Figure 2.4, panels C and F). Thus, the log-logistic model (Equation 5) is used throughout the rest of this study to parameterise single-particle intensity distributions.

The single-particle intensity distributions produced by u-track with GPU-fit are similar to those produced by u-track alone, but in general are less skewed and more precisely follow the log-logistic distribution (Figure 2.4, panels C and F vs. panels B and E). A likely explanation is that with GPU-fit every candidate spot is fit to only one Gaussian, while with u-track a candidate spot sometimes is fit to two or more Gaussians, which results in more particles with a lower average intensity. GMimPro detected fewer particles in total, and their intensity is less skewed to the right (Figure 2.4, panels A and D) when compared to both u-track and u-track with GPU-fit. Since GMimPro locates single particles by comparing the goodness-of-fit of each region in the image to an idealised fluorophore centroid, and since this centroid is modelled to resemble a single fluorophore, spots that contain multiple fluorophores and form a broader distribution have a higher likelihood of being rejected. Thus, GMimPro may be biasing the results to spots that resemble a single fluorophore and have a lower intensity.
Based on these results, we concluded that u-track with GPU-fit is the best single-particle detection and tracking algorithm, and that the log-logistic model is best model for fitting single-particle intensity distributions. GMimPro is biased in its detection of single particles, and u-track incorrectly assumes Gaussian noise. While attempting to fit candidate spots to multiple 2-dimentional Gaussians may improve the software’s ability to discern multiple fluorophores, the fact that the single-particle intensity distributions do not lose their heavy tail with photobleaching suggests that other physical phenomena may be involved in generating the shape and amplitude of the candidate spots. Those phenomena may not be captured correctly by multiple Gaussians, in the same way that the single-particle intensity distributions are not modelled correctly by a sum of two or more constrained Gaussians.
Figure 2.3: Images of a single cell expressing SNAP-CD86 labelled with SNAP-Surface 488. Panels A and B show an image of the cell before photobleaching. Panels C and D show an image of the same cell after 30 seconds of photobleaching. Panels A and C show raw images while B and D show background-corrected images displayed using an artificial colourmap. Green circles in panels B and D indicate spots that were detected and fit using u-track with GPU-fit.
Figure 2.4: Single-particle intensity distributions obtained by analysing timelapse images of a single cell expressing SNAP-CD86 using GMimPro, u-track and u-track with GPU-fit. Timelapse images of a single cell displayed in Figure 2.3 were analysed using GMimPro (panels A and D), u-track (panels B and E) and u-track with GPU-fit (panels C and F). Panels A to C show intensities of all single particles detected over a 20-frame time window before photobleaching while panels D to F show intensities of all single particles detected over a 20-frame time window after 30 seconds of photobleaching.

Figure 2.5: The raw intensity of a single candidate spot and the corresponding fit by a two-dimensional Gaussian. Panel A shows the intensity of the 7 x 7 pixel region around a putative single particle detected by u-track. Panel B shows the fit of a single Gaussian to the region in panel A, performed using GPU-fit.
2.4.3 Single-particle intensity distributions for CD28, CD86 and M2 receptor

After optimising the fluorophore labelling and image analysis techniques, we performed single-particle tracking experiments on cells expressing the M2 muscarinic receptor, CD28 and CD86. Representative images of cells expressing each protein labelled with SNAP-Surface 488 are shown in Figure 2.6 panels A to C. Single-particle intensity histograms obtained from a set of 30 frames before photobleaching and a set of 30 frames after 33 seconds of photobleaching are shown in Figure 2.6, panels D to F and G to I, respectively. The main factors determining the mean and standard deviation of the distributions are the level of expression and the average intensity of the images, and cells expressing monomeric CD86, dimeric CD28 and the M2 receptor at similar levels showed nearly identical single molecule intensity distributions. The mean of those distributions shifts to the left as the cells are photobleached, but the general shape remains the same, and is always skewed to the right. This is at variance with our prediction that the monomeric and dimeric controls would show single peaks centred at the intensity of a monomer or dimer, respectively.

In order to create a more representative sample, single-particle intensities obtained from a group of cells expressing a given protein were binned together, and are shown as histograms in Figure 2.7. Once again, those histograms appear similar for each protein, and retain the log-logistic shape before photobleaching (Figure 2.7, panels A, F and K) and after multiple successive 3.3 second bouts of photobleaching (Figure 2.7, panels B to E, G to J and L to O). The vertical dashed lines in each panel in Figure 2.7 correspond to the geometric means of the distributions. The geometric mean was used as an indicator of the average single-particle intensity throughout this study because, unlike the arithmetic mean, it is able to capture the skewed shape of the distribution.

The geometric mean of the single-particle intensity distribution before photobleaching is ~420 photons for CD86 and ~570 photons for CD28 (Figure 2.7, vertical lines in panels K and A, respectively). Thus, while single particles detected on cells expressing dimeric CD28 were on average brighter than the single particles on cells expressing monomeric CD86, they were not
twice as bright as would be expected when comparing a dimer to a monomer. Furthermore, due to the high cell-to-cell variability in average single-particle brightness, this ~150 photon difference was not statistically significant (p = 0.26). The average single-particle intensity for cells expressing the M2 receptor is ~540 (Figure 2.7, dashed line in panel F), but due to the high uncertainty in the data, no conclusion can be drawn from this value.

Since even an oligomeric protein would have at most one active fluorophore remaining after extensive photobleaching, it was expected that the average of the single-particle intensities eventually would converge to a constant value and would no longer be affected by photobleaching. However, the geometric means of the single-particle intensity distributions obtained for the M2 receptor, CD28 and CD86 decay exponentially as a function of photobleaching time (Figure 2.8, A), and continue to decrease after 16 seconds of photobleaching (Figure 2.8, A; frame 500) and even after 33 seconds of photobleaching (data not shown). Thus, while the broadness of the initial single-particle intensity distributions may come from random coincidence of several fluorescent spots in the same diffraction-limited volume, other processes also are likely to be involved.

When we plot the geometric mean of the single-particle intensity distribution as a function of the background image intensity (calculated by taking the $l_1$-norm of the image), we note that the two variables are highly correlated (Figure 2.8, panel B). It seems unlikely that the background intensity amplifies the intensity of single particles directly, as the background intensity already is taken into account through an intensity offset during the fitting of each candidate spot to a two-dimensional Gaussian. A more plausible explanation is that both the background intensity and the intensity of detected particles depend on a third factor, which decreases as a function of photobleaching time. One such factor could be the variability in the distance between the fluorophores and the glass/water interface, caused by irregularities in the glass surface and ruffles in the basal membrane of a cell. Fluorophores that are closer to the glass/water interface would be excited more strongly by the laser, would produce a stronger signal and would photobleach more quickly than fluorophores that are further away. Consequently, intensities recorded before photobleaching would be higher than intensities recorded after photobleaching,
as they would arise from molecules that are, on average, closer to the glass/water interface. This would be applicable both to the SNAP Surface dyes that are responsible for single-particle intensities and to autofluorescent molecules that are responsible for the background image intensity.

If the concentration of autofluorescent proteins is the same for all cells, it is reasonable to compare average single-particle intensities for different constructs by extrapolating the values to the same background intensity. At a background intensity of 200 photons per pixel (Figure 2.8, panel B, vertical line), the average single-particle intensity of the M₂ receptor is predicted to be 618 photons, the average single-particle intensity of CD28 is predicted to be 586 photons and the average background intensity of CD86 is predicted to be 470 photons. The single-particle intensity of the dimeric control CD28 is only marginally higher than the single-particle intensity of the monomeric control CD86, and the difference is not significant given the degree of variation from cell to cell. This suggests that even by extrapolating to a constant background value, a dimer cannot be distinguished from a monomer using our analysis.

We also tried to look at the intensities of individual particles over time. In the case of a monomer, it was expected that most particles would photobleach in a single step, while in the case of a dimer, it was expected that many particles would photobleach in two steps. Single-particle intensity tracks obtained from cells expressing CD86, CD29 and the M₂ receptor are presented in panels A, B and C in Figure 2.9. If we assume that the intensity of a single fluorophore is around 400 photons, which appears to be consistent with most of the tracks presented in Figure 2.9, then all three proteins show approximately the same number of single photobleaching steps (as in Figure 2.9, panel A[3,4]), double photobleaching steps (as in Figure 2.9, panel C[1,1]) and merge-split behaviours (as in Figure 2.9, panel B[3,2]). Furthermore, many of the single-particle tracks show a gradual decline in intensity rather than abrupt photobleaching (as in Figure 2.9, panel C[3,3]). We could not find a distinct trend that would allow us to separate the monomeric control CD86 from the dimeric control CD28 based on the temporal trajectories of single-particle intensities. Consequently, we could not determine the oligomeric size of the M₂ receptor.
Figure 2.6: Representative TIRF microscopy images and single-particle intensity distributions obtained from cells expressing SNAP-CD86, SNAP-CD28 and SNAP-M2. Panels A, B and C show sample TIRF microscopy images of cells expressing SNAP-CD86, SNAP-CD28 or SNAP-M2, respectively, and panels D and G, E and H, and F and I show the corresponding single-particle intensity distributions. Panels D to F show the single-particle intensity distributions obtained before photobleaching, while panels G to I show single-particle intensity distributions obtained after 33 seconds of photobleaching.
Figure 2.7: Single-particle intensity distributions obtained from multiple cells expressing SNAP-CD86, SNAP-CD28 and SNAP-M2 before, during and after photobleaching. Panels A to E show single-particle intensity distributions produced using all single particles that were detected in 9 cells expressing SNAP-CD28. Panels F to J show single-particle intensity distributions produced using all single particles that were detected in 5 cells expressing SNAP-M2. Panels K to O show single-particle intensity distributions produced using all single particles that were detected in 3 cells expressing SNAP-CD28. Single-particle intensity distributions in column A to K were obtained from 10 frames before photobleaching, while single-particle intensity distributions in columns B to L, C to M, D to N and E to O were obtained from 10 frames after 3.3 seconds, 6.6 seconds, 10 seconds and 13.3 seconds of photobleaching, respectively. Each single-particle intensity distribution was fit by the log-logistic function (black curves), and the geometric mean of each distribution is indicated with a vertical line.
Figure 2.8: Geometric means of the single-particle intensity distributions plotted as a function of photobleaching time and mean background intensity. Geometric mean of the single-particle intensity distributions (vertical lines in Figure 2.7) decrease exponentially as a function of photobleaching time (panel A) and linearly as a function of the background image intensity (panel B).
Figure 2.9: Temporal trajectories of single particle intensities for cells expressing SNAP-CD28, SNAP-CD86 and SNAP-M2. Panels A, B and C show temporal trajectories of single-particle intensities for 16 particles selected from cells expressing SNAP-CD86, SNAP-CD28 and SNAP-M2, respectively. The horizontal dashed lines correspond to the approximate intensity of a single fluorophore (400 photons) and of two fluorophores (800 photons).
2.5 Discussion

2.5.1 Summary of the results

We used single-particle tracking to try to measure the oligomeric size of M2 muscarinic receptor. Unfortunately, the results that we obtained, both from the single-particle intensity distributions and the trajectories of the single-particle intensities over time, are ambiguous. We could not reliably distinguish the monomeric control CD86 from the dimeric control CD28, and so we cannot make any conclusions regarding the oligomeric size of the M2 receptor. It seems likely that the signal-to-noise ratio of our images was not sufficiently high to discern single particles from noise, and this made all further analysis futile.

In order to measure accurately the intensity and the number of photobleaching steps, the M2 receptor and the CD28 and CD86 reference controls had to be labelled with a bright and photostable synthetic dye. However, it was difficult to ensure complete labelling of the proteins with an exogenous dye while at the same time maintaining very little non-specific adsorption of that dye to the cell surface and the coverslip. Moreover, the brightness and photostability of individual dyes was variable and the number, and intensity of detected particles would change depending on the software and parameters used during the analysis of the images (Figure 2.4), which made it difficult to interpret the data. We tried to perform SPT experiments with GFP-labelled proteins, but since fluorescent proteins are neither bright nor photostable, the images that we obtained were nearly impossible to analyse (data not shown). Consistent with these results, the only study that had quantified the number of photobleaching steps for GFP-labelled proteins expressed in live cells used a special 1.65 numerical aperture objective and high refractive index coverslips [68], which are not available in most imaging facilities.
2.5.2 Skewed single-particle intensity distributions

Single-particle intensity distributions obtained for CD28, CD86 and the M2 receptor were always skewed to the right and were fit best by the log-logistic distribution. Many other SPT studies have reported similar skewed distributions [52,53,69,70]. However, in most of those studies, the distributions were fit to a sum of Gaussians, and the proportions of monomers and various oligomers were determined from the relative areas of the corresponding Gaussians. Since the distributions that we obtained had the same skewed shape both before photobleaching and after an extensive period of photobleaching, at least in our case this type of analysis appears to be incorrect. Two other groups have reached a similar conclusion, and in an attempt to account for the persistent skewness of the distributions, they proposed two alternative ways of analysing the data.

Meckel et al. [71] examined eYFP localised to the plasma membrane with a dual palmitoylation and prenylation sequence from H-Ras (eYFP-C10HRAS) and reported that, on their confocal setup, all of eYFP photobleached within one 50 ms frame. The authors derived a function that models the distribution of intensities for single molecules that photobleach within one frame (Equation 6), and used successive autoconvolutions of this function to model the distribution of intensities for two or more such molecules colocalised within a diffraction-limited spot (Equation 7). In the above equations, \( n \) is the number of photons detected for a single molecule, \( N \) is the average number of photons detected for single molecules and \( k \) is the number of single molecules colocalised within a diffraction-limited spot. The authors used a sum of Equations 6 and 7, together with a term that accounts for the higher probability of detecting brighter spots as opposed to dimmer spots, to fit the skewed single-particle intensity distributions that they obtained for eYFP-C10HRAS. This provided them with an estimate of the number of monomers, dimers and higher-order oligomers of eYFP-C10HRAS present at a given level of expression. The authors had to use expression levels of less than 0.2 molecules per \( \mu m^2 \) in order to obtain single-particle intensity distributions that matched what was expected for a monomer, and even at expression levels of 0.25 molecules per \( \mu m^2 \) only 14 % of the resolved spots could be attributed to a single eYFP-C10HRAS, highlighting the importance of low expression levels in SPT experiments.


\[ p_l(n; N) = \frac{1}{N} \left( 1 + \frac{1}{N} \right)^{-(n+1)} \]

\[ p_k(n; N) = \sum_{n'<0}^{\infty} p_l(n-n') p_{k-1}(n') \]

Mutch et al. [72] have reported that single-particle intensity distributions obtained from single Alexa Fluor 488 molecules, from antibodies labelled with multiple Alexa Fluor 488 molecules and from multiple antibodies labelled with multiple Alexa Fluor 488 molecules, all have a similar shape that is well-approximated by a log-normal distribution. If the log-normal shape of those distributions was due to the photophysics of single Alexa Fluor 488 molecules, then the distributions would become more Gaussian-shaped as more fluorophores are used to label an object, and this was not observed. Thus the authors conclude that the log-normal shape is caused by the multiplication of various measurement artefacts that are intrinsic to the TIRF imaging process. Those artefacts could include mild defocusing in various regions of the image, spatial heterogeneities in the intensity of the illuminating evanescent wave, variations in the pixel quantum efficiency in the CCD camera, dirt and aberrations in the optics and ruffles in the basal membrane of a cell that puts fluorophores different distances away from the exponentially-decaying evanescent field [72].

It appears, therefore, that if the protein of interest is labelled with a fluorescent proteins or a photounstable organic fluorophore, the single-particle intensity distributions may appear non-Gaussian because most fluorophores will have photobleached by the end of a single frame. However, even if a stable fluorophore or an ensemble of fluorophores is used to label the protein, single-particle intensity distributions still may be non-Gaussian, and the underlying principles responsible for the skewness of the distributions remain poorly defined.
2.5.3 Recent single-particle tracking studies

During the preparation of this manuscript, Calebiro et al. published a study that used SPT to measure the oligomeric size of the β₁ and β₂ adrenergic receptors, and the GABA<sub>B1</sub> / GABA<sub>B2</sub> receptor heterodimer [39]. Similar to our own work, Calebiro et al. used SNAP-tag technology to label their receptors, and they used CD86 fused to one or two SNAP-tags as the monomeric and dimeric controls. The authors labelled the SNAP-tagged proteins with Alexa Fluor 647, which seemed to result in better signal-to-noise images with much less cell autofluorescence than what we were able to achieve with SNAP-Surface 488. Both the β₁ and β₂ adrenergic receptors were found to exist as a mixture of monomers and dimers, which is similar to the results obtained previously for the M₁ muscarinic receptor and the N-formyl peptide receptor [52,53]. The GABA<sub>B1</sub> / GABA<sub>B2</sub> receptor heterodimer was found to exist as a mixture of dimers and tetramers. While Calebiro et al. fit their single-particle intensity distributions to a sum of Gaussians, they nonetheless were able to calculate accurately the oligomeric size of their monomeric and dimeric controls, and their results from the single-particle intensity distributions are in close agreement with the results they obtained by measuring photobleaching steps. Therefore, it appears that fitting single-particle intensity histograms to a sum of Gaussians still may be an adequate approach, especially when differentiating between monomers and dimers.

In another paper published during the preparation of this manuscript, Nenasheva et al. used SPT to measure the oligomeric size of the M₂ muscarinic receptor [61]. The authors found that M₂ receptors exist as a mixture of monomers and reversible dimers in CHO cells, in cardiac cell lines (HL-1) and in primary cardiomyocytes. This study did not use monomeric or dimeric controls, and M₂ receptors were labelled with Cy3B-telenzepine, which would not have labelled all sites in a negatively cooperative oligomer. Nevertheless, their conclusion is consistent with the three SPT studies discussed above, which did not have those shortcomings.
2.5.4 Conclusion

In total, there are now four SPT studies that analysed the oligomeric size of five class A GPCRs, and all of those studies have concluded that the receptors exist as a mixture of monomers and transient dimers. Even though our attempts at using SPT to measure the oligomeric size of M2 receptors were unsuccessful, the studies performed by Calebiro et al. and Nenasheva et al. have made this work largely unnecessary. It is likely that if we used Alexa-fluor 647 instead of SNAP Surface-488 to label our proteins, and CD86 with two SNAP-tags linked in tandem instead of CD28 as our dimeric control, we too would have observed that M2 receptors exist as a mixture of monomers and dimers in live CHO cells. Furthermore, since SPT has a resolution limit of ~ 200 nm, the dimers observed in SPT studies may in fact be monomers that are colocalised in microdomain smaller in size than the diffraction limit. One possible future study would be to used super-resolution imaging or FRET to measure the distance between protomers in those apparent dimers and distinguish true dimers from co-localised molecules.
Chapter 3
Förster resonance energy transfer
3 Förster resonance energy transfer

3.1 Abstract

Förster resonance energy transfer (FRET) is a specific and sensitive technique for detecting the oligomerisation of cytosolic proteins, but using FRET to measure the oligomerisation of membrane proteins is more challenging. Membrane proteins are confined to two dimensions. Many of them cluster in small microdomains, which can lead to spurious FRET at high levels of expression. Those challenges have become apparent in the study of G protein-coupled receptors (GPCRs), where different groups have used FRET efficiencies to conclude variously that class A GPCRs exist as monomers, dimers or tetramers. To examine what led to such markedly different conclusions, we measured FRET efficiencies for membrane proteins that are known to exist as monomers, transient oligomers or stable dimers; we then compared the patterns of efficiencies observed for those controls to the patterns obtained for the M2 muscarinic receptor, a prototypical class A GPCR. In each case, proteins were tagged with GFP2 and eYFP and were expressed transiently in CHO or HEK cells. Stable and constitutive oligomers produced efficiencies that remained high even at low levels of expression and could be described well by a model for a stable dimer. In contrast, efficiencies measured between monomeric CD86 molecules, between M2 receptors, between M2 receptors and Gα1 proteins, and between M2 receptors and M1 receptors, β2 receptors, CD28 or CD86, all followed a similar pattern that approached zero at low levels of expression and could not be described by a model for a stable dimer. That pattern could be described, however, by a model for bystander FRET or a transient dimer. The view of class A GPCRs as transient oligomers appears to be the most consistent with other biochemical and biophysical data that have been used to quantify GPCR oligomerisation and hitherto have led to a variety of contrasting interpretations.
3.2 Introduction

Förster resonance energy transfer (FRET) is non-radiative energy transfer that occurs through dipole-dipole interactions between two chromophores that are in close proximity and share an overlap in their emission and excitation spectra. Measuring FRET between proteins tagged with organic fluorophores or synthetic dyes has become a widely-used approach for detecting protein oligomerisation. FRET can also occur with *Renilla* luciferase rather than a laser-excited fluorophore as the energy donor, and this technique is often referred to as BRET. This eliminates the need for laser excitation but results in a much weaker signal. While the presence of non-trivial amounts of FRET between cytosolic proteins typically implies oligomerisation, FRET between membrane proteins is more difficult to interpret. Membrane proteins are confined to two dimensions, and many of them cluster in small microdomains. Even monomeric membrane proteins can yield high FRET efficiencies, as was observed between myristoylated palmitoylated mCFP and mYFP [73] over 10 years ago, and determining whether the observed FRET arises from non-specific clustering, transient association or constitutive oligomerisation of the membrane protein remains a challenge.

Despite the difficulty in interpreting the results, FRET measurements have been used extensively to study class A G protein-coupled receptor (GPCRs), and in most of those cases they were used to support the existence of receptor homo- or hetero-oligomers. To list a few examples, the A1 adenosine receptor [54] and the β1 and β2 adrenergic receptors [55] were shown by FRET to be exclusively dimers, the δ and κ opioid receptors [58], the CCR5 chemokine receptors [56] and M3 muscarinic receptors were shown to be dimers or higher-order oligomers [57], the M2 muscarinic receptors [60] were shown to be tetramers and the D2 dopamine receptors [59] were shown to be tetramers or higher-order oligomers. A more complete set of studies that used FRET to show the existence of GPCR oligomer can be found in the GPCR oligomerisation knowledge base [74].
Only two FRET-based studies, examining the neurokinin-1 receptor [75] and the β2 adrenergic receptor [76], had concluded that the receptors were monomeric. But James et al. [76] suggested that the high FRET efficiencies observed in the other studies were due to non-specific interactions between over-expressed receptors. They performed two types of experiments that, they claim, allowed them to distinguish between monomers, transient dimers and stable dimers. In the “type I” experiment, FRET efficiencies were measured as a function of acceptor-to-donor ratio while the total expression of acceptors and donors was kept constant. The FRET efficiencies of the monomeric control CD86 and the β2 adrenergic receptor reached an asymptote and did not change when the acceptor-to-donor ratio was greater than 2, while FRET efficiencies of the transient dimer CD80 and stable dimers CD28 and CTLA-4 were significantly higher and maintained the hyperbolically increasing trend up to the acceptor-to-donor ratio of 10. In the “type II” experiment, FRET efficiencies were measured as a function of total acceptor and donor expression while the acceptor-to-donor ratio was kept constant. The FRET efficiencies of the monomeric controls CD2 and CD86 and the FRET efficiencies of the β2 adrenergic receptor approached zero at low levels expression, while the FRET efficiencies of the dimeric control CTLA-4 remained high and were largely independent of the expression level. James et al. used those findings to conclude that the β2 adrenergic receptor cannot be a stable or transient dimer and is exclusively a monomer, and they speculate that the other studies that measured FRET efficiencies between class A GPCRs to show the existence of homo- and hetero-oligomers had misinterpreted their results.

Experiments conducted by James et al. to distinguish between monomers, transient oligomers and stable dimers have limitations, and many of those limitations were stressed in subsequent correspondences [77,78]. We also note that, according to the mathematical model developed by Wolber and Hudson in 1979 [79], the apparent FRET efficiency between randomly-dispersed monomers depends only on the acceptor expression level and not on the donor expression level. Thus, if the total amount of donors and acceptors is held constant, as was done by James et al. in their “type I” experiment, the acceptor expression level will increase in accordance with the acceptor-to-donor ratio, which will lead to an increase in the apparent FRET efficiency even between non-interacting molecules. James et al. propose that, if the total concentration of donors
and acceptors is held constant, the FRET efficiency for randomly-dispersed monomers should remain constant at acceptor-to-donor ratios greater than two, since each donor will “experience the same acceptor environment”. This is incorrect, since for instance at an acceptor-to-donor ratio of 10 to 1, the concentration of acceptors is 1.36 times higher at an acceptor-to-donor ratio of 2 to 1. Furthermore, for “type I” experiments James et al. assume that the total expression level is the same at all acceptor-to-donor ratios for all proteins being investigated, and for “type II” experiments they assume that the acceptor-to-donor ratio is the same at all expression levels for all proteins being investigated. In neither case do they have direct evidence in support of those assumptions. Salahpour and Masri [78] note that the acceptor-to-donor ratio often is correlated with the expression level, and this coincides with our own observations. Furthermore, we note that different proteins can show a different acceptor-to-donor ratio and a different expression level even when the cells are transfected with the same fraction of acceptor- and donor-expressing plasmids and for the same period of time.

The purpose of this study was to revisit the conclusions made by James et al. and other groups and try to resolve the controversy surrounding FRET measurements between GPCRs. We used the M2 muscarinic receptor as the prototypical class A GPCR, and we used CD86 as the monomeric control and CD28 and caveolin-1 as oligomeric controls. In addition, we measured FRET efficiencies between M2 receptors and Gαi1 proteins, and between M2 receptors and M1 receptors, β2 receptors, CD28 and CD86 (Figure 3.11). We tagged all proteins with GFP2 and eYFP, and used a spectrally-resolved microscope that allowed us to measure the FRET efficiency and the amount of donor and acceptor present in each cells. Four different models were employed to assist with the interpretation of the results. The bystander FRET model, derived by Wolber and Hudson [79], predicts the effect of the acceptor expression level on the apparent FRET efficiency between randomly-dispersed molecules. The stable dimer model, derived by Veatch and Stryer [80] and amended by Raicu [81], predicts the effect of the acceptor-to-donor ratio on the apparent FRET efficiency between stable dimers and higher-order oligomers. The stable dimer with bystander FRET model and the transient dimer model were derived in this work, and predict the effect of both the acceptor expression level and the
acceptor-to-donor ratio on the apparent FRET efficiency observed for a mixture of monomers and stable dimers, or for transient dimers, respectively.

In order to overcome the shortcomings of the James et al. study, we present the FRET efficiency, the acceptor-to-donor ratio, and the acceptor expression level for every single cell or data point. We show that keeping either the acceptor-to-donor ratio or the acceptor expression level constant while varying the other parameter is nearly impossible, and the two parameters often are highly correlated. This complicates the interpretation of the results, since the bystander FRET model and the transient oligomer model share a similar dependence on the acceptor expression level and acceptor-to-donor ratio, respectively. We stress that, in order to be able to distinguish between monomers and stable dimers, it is essential that estimates of the acceptor expression level and acceptor-to-donor ratio accompany every single FRET measurement.

Even with our more complete way of presenting and analysing the data, we find that the pattern of FRET efficiencies obtained for the M2 muscarinic receptor is inconsistent with the view that this receptor exists as a constitutive oligomer in live cells, which is similar to the conclusion made by James et al. for the β2 adrenergic receptor. However, we show that it is very difficult to discern between monomers, transient oligomers, and a combination of monomer and oligomers based on FRET measurements alone, and the FRET data suggests that M2 muscarinic receptors may exist as monomers, as transient oligomers, or as monomers with a small subpopulation of oligomers. Recent single-particle tracking experiments, performed at a low level of expression, show that class A GPCR monomers can come together to form transient complexes in a concentration-dependent manner [39,52,53,61]. This view of class A GPCRs as transient oligomers appears to be the most consistent with the biochemical and biophysical data that have been used to quantify GPCR oligomerisation and hitherto have led to a variety of contrasting interpretations.
3.3 Mathematical models

3.3.1 Acknowledgements

Most of the theory describing the calculation of donor and acceptor concentrations and apparent FRET efficiencies from spectral images, as well as the models that we used to interpret the FRET data, were developed by Valerică Raicu and have been described previously [60,81,82]. The model for bystander FRET was derived in a seminal work by Wolber and Hudson [79]. What follows is a brief synopsis of those techniques and models, with an emphasis on their application to this project.

3.3.2 Measuring FRET efficiencies

Multiple techniques have been developed to measure and quantify FRET, including donor dequenching (Equation 1), acceptor-sensitised emission (Equation 2), and time and frequency domain fluorescence lifetime imaging (FLIM) (Equation 3). Here, \( E_{app}^{Dq} \), \( E_{app}^{Ase} \), and \( E_{FLIM} \) are the apparent FRET efficiencies obtained through donor dequenching, acceptor-sensitised emission and FLIM, respectively. \( F_D(\lambda_{ex}) \) and \( F_A(\lambda_{ex}) \) are the photon emission from donors and acceptors excited alone, \( F_{DA}(\lambda_{ex}) \) and \( F_{AD}(\lambda_{ex}) \) are the photon emission from donors in the presence of acceptors and from acceptors in the presence of donors, and \( F_D(RET) \) and \( F_A(RET) \) are the photons lost by the donors and gained by the acceptors through resonance energy transfer (RET).

\[
\begin{align*}
E_{app}^{Dq} &= \frac{F_D(RET)}{F_D(\lambda_{ex})} = 1 - \frac{F_{DA}(\lambda_{ex})}{F_D(\lambda_{ex})} \\
E_{app}^{Ase} &= \frac{F_A(RET)}{F_A(\lambda_{ex})} = \frac{F_{AD}(\lambda_{ex})}{F_A(\lambda_{ex})} - 1
\end{align*}
\]
\[ E^{FLIM} = 1 - \frac{\tau_{DA}}{\tau_D} \]

\( F_{DA}(\lambda_{ex}) \) is smaller than \( F_D(\lambda_{ex}) \) because donors lose some excitation energy through RET (Equation 4), while \( F_{AD}(\lambda_{ex}) \) is bigger than \( F_A(\lambda_{ex}) \) because acceptors gain some excitation energy through RET (Equation 5). If the donor and acceptor are properly chosen and the donor has a large Stokes shift, \( F_A(\lambda_{ex}) \) will be nearly zero at the \( \lambda_{ex} \) used in the experiment, and Equation 5 reduces to Equation 6. The number of photons lost by the donor through RET (\( F_D(RET) \)) will be equal to the number of photons gained and emitted by the acceptor (\( F_A(RET) \) or \( F_{AD}(\lambda_{ex}) \)) normalized by the ratio of the donor and acceptor quantum yields (Equation 7).

\[
F_{DA} = F_D(\lambda_{ex}) - F_D(RET) \tag{4}
\]

\[
F_{AD} = F_A(\lambda_{ex}) + F_A(RET) \tag{5}
\]

\[
F_{AD} = F_A(RET) \tag{6}
\]

\[
F_D(RET) = \frac{Q^D}{Q^A} F_A(RET) = \frac{Q^D}{Q^A} F_{AD} \tag{7}
\]

Spectral images obtained using a spectrally resolved microscope (Equation 8) can be unmixed to obtain the values for the maximum emission intensities for the donor in the presence of acceptor \( k_{DA} \) and acceptor in the presence of donor \( k_{AD} \) (Equations 9 and 10, respectively).

\[
F_M(\lambda) = k_{DA} F_D(\lambda) + k_{A} F_A(\lambda) \tag{8}
\]

\[
k_{DA} = \frac{\sum (F_M \cdot F_D) \sum (F_A^2) - \sum (F_A \cdot F_D) \sum (F_M \cdot F_A)}{\sum (F_A^2) \sum (F_D^2) - [\sum (F_A \cdot F_D)]^2} \tag{9}
\]
Using the values for $k_{DA}$ and $k_{AD}$, and the elementary spectral integrals for donors ($w_D$) and acceptors ($w_A$), we can calculate the total photon emission from donors in the presence of acceptors ($F_{DA}$) and acceptors in the presence of donors ($F_{AD}$) (Equations 11 and 12).

\[
F_{DA} = k_{DA} \int_{\lambda_{em}} i_D(\lambda_{em})d\lambda_{em} = k_{DA}w_D
\]

\[
F_{AD} = k_{AD} \int_{\lambda_{em}} i_A(\lambda_{em})d\lambda_{em} = k_{AD}w_A
\]

Finally, using the equation for apparent FRET efficiency obtained through donor dequenching (Equation 1), we can calculate the apparent FRET efficiency for each cell (Equation 13).

\[
E^{\text{app}}_{Dq} = \frac{F_D(RET)}{F_D(\lambda_{ex})} = \frac{1}{1 + \frac{Q_Ak_{DA}w_D}{Q_Dk_{AD}w_A}}
\]

The intensity of the donor in the absence of the acceptor $k_D$ can be obtained by correcting the intensity of the donor in the presence of the acceptor $k_{DA}$ by the amount of energy the donor lost through FRET (Equation 14).

\[
k_D = \frac{k_{DA}}{1 - E^{\text{app}}_{Dq}}
\]

The intensity of the acceptor in the absence of donor $k_A$ can be obtained by taking a second image using an excitation wavelength that excites only the acceptor and measuring the intensity of the acceptor at the wavelength corresponding to its emission maximum.

Using the Beer-Lambert law and the values for $k_D$ and $k_A$, it is possible to calculate the total concentrations of donors and acceptors present in the cell in units that are arbitrary but equal for
both the donor and the acceptor (Equations 15 and 16). In these Equations \( \varepsilon_D \) and \( \varepsilon_A \) are the molar extinction coefficients of the donor and the acceptor, \( W^{405} \) and \( W^{514} \) are the laser powers at the wavelengths used to excite the donor and the acceptor, and \( e_D^{405} \) and \( e_A^{514} \) are the correction factors that account for suboptimal excitation of donor and acceptor at those wavelengths.

\[
[D]_i = \frac{k_D}{Q_D \varepsilon_D e_D^{405} W^{405}} \\
[A]_i = \frac{k_A}{Q_A \varepsilon_A e_A^{514} W^{514}}
\]

### 3.3.3 Stable dimer model

The dependence of the apparent FRET efficiency on the donor and the acceptor mole fractions (\( P_D \) and \( P_A \), respectively) has been derived in [81], and simplifies to Equation 17 if we assume that the distance between each donor and acceptor in a complex is the same. Here, \( n \) is the number of protomers in an oligomer, \( k \) is the number of donors and \( (n-k) \) is the number of acceptors.

\[
E_{\text{app}}^{\text{DL}} = \frac{1}{n} \sum_{k=1}^{n-1} \frac{k(n-k)E_{\text{pairwise}}}{1 + (n-k-1)E_{\text{pairwise}}} \left( \frac{n}{k} \right) \left( p_D^{k-1} P_A^{n-k} \right)
\]

In the case of a dimer, Equation 17 simplifies to Equation 18, which can be re-written as Equation 19 to highlight the dependence of the apparent FRET efficiency on the acceptor-to-donor ratio ([\( A_i \) / [D]_i]) rather than on the acceptor mole fraction (\( P_A \)).

\[
E_{\text{stable dimer}} = E_{\text{pairwise}} \cdot P_A
\]
In the case of a mixture of monomers and dimers, the apparent FRET efficiency is given by Equation 20, which has the same shape as Equation 18 but is scaled by $\mu$: the fraction of protomers that exist as dimers.

$$E_{\text{mixture of monomers and stable dimers}} = \frac{\mu \cdot E_{\text{pairwise}}}{1 + \frac{[A]_i}{[D]_i}}$$

If any of the constants in Equations 15 and 16 are not accurate, the calculated acceptor-to-donor ratio will be off by some multiple for all cells that are imaged. To account for this possibility, an extra variable $c$ can be introduced into Equation 19 and allowed to vary within a reasonable range (Equation 21).

$$E_{\text{stable dimer with correction}} = \frac{E_{\text{pairwise}}}{1 + \frac{[A]_i}{[D]_i} \cdot c}$$

Equation 19 takes a more complicated form for higher order oligomers (Equation 17), but the dependence of the apparent FRET efficiency on the acceptor and donor mole fractions is nearly identical for a dimer and a higher order oligomer, if an appropriately smaller value for $E_{\text{pairwise}}$ is chosen for the oligomer [60]. In order to illustrate this, we simulated apparent FRET efficiencies as a function of acceptor-to-donor ratio for a mixture of 40% monomers and 60% dimers with an 80% pairwise FRET efficiency (i.e. Equation 20 with $\mu = 0.60$ and $E_{\text{pairwise}} = 0.80$; Figure 3.1, black line). We fit the simulated data to models for a stable dimer (Equation 19; Figure 3.1, cyan line), a stable trimer (Equation 17 with $n = 3$; Figure 3.1, red line), a stable tetramer (Equation 17 with $n = 4$; Figure 3.1, green line) and a stable hexamer (Equation 17 with $n = 6$; Figure 3.1, blue line). The curves for a transient dimer and a stable dimer are superimposable, and the curves for a stable trimer, tetramer and hexamer also are very close. Thus Equation 19 can be used to fit
the data for a stable oligomer of any size, as long as one remembers that the value for $E_{\text{pairwise}}$ is valid only if the protein is a dimer.
Figure 3.1: Comparison of the models for a stable dimer, a stable trimer, a stable tetramer and a stable hexamer to the model for a mixture of monomers and stable dimers. FRET efficiencies were simulated for a mixture of 40% monomers and 60% dimers with an 80% pairwise FRET efficiency (i.e. Equation 20 with $\mu = 0.60$ and $E_{\text{pairwise}} = 0.80$; black line). The simulated data were fit to a model for a stable dimer (Equation 19; cyan line), a stable trimer (Equation 17 with $n = 3$; red line), a stable tetramer (Equation 17 with $n = 4$; green line) and a stable hexamer (Equation 17 with $n = 6$; blue line). The pairwise FRET efficiency predicted by each model is different ($E_{\text{pairwise}}$ given in the legend), but the general dependence on the acceptor-to-donor ratio is the same.
3.3.4 Transient dimer model

In the previous section, we presented a model that describes the relationship between the apparent FRET efficiency and the acceptor-to-donor ratio for a mixture of monomers and dimers (Equation 20). This model assumes that the fraction of protomers that exist as dimers ($\mu$) is constant and does not depend on the total level of expression. However, if monomers and dimers exist in dynamic equilibrium, this assumption is not correct.

The proportion of monomers and dimers present in the system at equilibrium can be described by Equation 22, where $K_{eq}$ is the equilibrium association constant, $C_{total}$ is the total concentration of donor and acceptor protomers, and $\mu$ is the fraction of protomers that exist as dimers.

$$K_{eq} = \frac{\mu \cdot C_{total}}{((1 - \mu) \cdot C_{total})^2}$$

Equation 22 can be solved for $\mu$ using the quadratic formula, and only the minus in the $\pm$ sign in the quadratic formula is physically relevant (Equation 23).

$$\mu = 1 + \frac{1}{4C_{total}K_{eq}} - \sqrt{1 + \frac{8C_{total}K_{eq}}{4C_{total}K_{eq}}}$$

A model that describes the apparent FRET efficiency for a mixture of monomers and dimers that are in dynamic equilibrium (Equation 24) is obtained by substituting Equation 23 into Equation 20. Throughout this work, we refer to this model as the “transient dimer” model.

$$E_{\text{transient dimer}} = \left(1 + \frac{1}{4C_{total}K_{eq}} - \sqrt{1 + \frac{8C_{total}K_{eq}}{4C_{total}K_{eq}}} \right) \cdot E_{\text{pairwise}}$$

Equation 24 can be simplified further to produce Equation 25.
Equation 25 can be expressed as a function of total acceptor expression ([A]_t) and acceptor-to-donor ratio ([A]_t/[D]_t) (Equation 26). This allows us to plot the transient dimer model on the same set of coordinates as the bystander FRET model (Equation 32), which is a function of [A]_t, the stable dimer model (Equation 19), which is a function of [A]_t/[D]_t, and the stable dimer with bystander FRET model (Equation 41), which is a function of both [A]_t and [A]_t/[D]_t.

\[
E_{\text{transient dimer}} = \frac{1}{1 - \sqrt{1 + 8C_{\text{total}}K_{eq}}} \cdot \frac{E_{\text{pairwise}}}{1 + \frac{1}{[A]_t/[D]_t}} \cdot \frac{1}{1 - \sqrt{1 + 8[A]_t \cdot \left(1 + \frac{1}{[A]_t/[D]_t}\right) \cdot K_{eq}}} \cdot \frac{1}{1 + \frac{1}{[A]_t/[D]_t}}
\]
3.3.5 Transient oligomer model

We could not find an analytical solution describing the formation of tetramers or higher-order oligomers. However, if we think of a tetramer as a combination of two dimers, and of a hexamer as a combination of a dimer and a tetramer, then the formation of a transient hexamer can be described using the schematic shown below. Here, $k_1$, $k_3$ and $k_5$ are the association rate constants, $k_2$, $k_4$ and $k_6$ are the dissociation rate constants and the ratios $k_1 / k_2$, $k_3 / k_4$ and $k_5 / k_6$ are equal to the equilibrium association constants $K_{eq1}$, $K_{eq2}$ and $K_{eq3}$.

The change in the concentrations of monomers, dimers, tetramers and hexamers can be described using the differential equations presented in Equation 27. The Jacobian matrix for this set of equations is presented in Equation 28.

\[
\begin{align*}
\frac{d[M]}{dt} &= -2k_1[M]^2 + 2k_2[MM] \\
\frac{d[MM]}{dt} &= k_1[M]^2 - k_3[MM] - 2k_1[MM]^2 + 2k_4[MMMM] - k_5[MM][MMMM] + k_6[MMMMMM] \\
\frac{d[MMMM]}{dt} &= k_2[MM]^2 - k_4[MMMM] - k_3[MM][MMMM] + k_5[MMMMMM] \\
\frac{d[MMMMMM]}{dt} &= k_5[MM][MMMM] - k_6[MMMMMM]
\end{align*}
\]
By integrating the above equations to equilibrium, we can find the equilibrium concentrations of monomers, dimers, tetramers and hexamers at any total concentration of protomers. Using Equation 29, we can predict the apparent FRET efficiency that would be observed at those concentrations, at a given acceptor-to-donor ratio. Here, $n$ is the number of protomers in an oligomer, $k$ is the number of donors, $(n-k)$ is the number of acceptors, $P_D$ is the donor mole fraction, $P_A$ is the acceptor mole fraction and $C_n$ is the concentration of species of size $n$ (i.e. $C_1 = [M], C_2 = [MM], \text{etc.}$).

$$E_{\text{mixture of monomers, dimers, tetramers and hexamers}} = \sum_{n=1}^{6} \frac{C_n \sum_{k=1}^{n-1} \frac{k(n-k)E_{\text{pairwise}}(n)}{1 + (n-k-1)E_{\text{pairwise}}(n)}}{\sum_{n=1}^{6} C_n}$$

In order to measure how well the model for a transient dimer can describe the pattern of efficiencies produced by a transient hexamer, we used the transient hexamer model to simulate FRET efficiencies at concentrations ranging from 0 to 26 A. U., and we fit those patterns of efficiencies using the model for a transient dimer. We performed 125 simulations, with each of the three equilibrium association constants ($K_{eq1}, K_{eq2}$ and $K_{eq3}$) set to 0.001, 0.1, 1, 10 or 1000. In each simulation, the pairwise FRET efficiency was set to 0.5 and the acceptor-to-donor ratio was set to 10. The concentration range of 0 to 26 A. U. was chosen so that at the intermediate concentration of 13 and at the intermediate equilibrium association constant of 1, the concentration of monomers, dimers, tetramers and hexamers is equivalent and is equal to 1. By performing simulations above and below those intermediate values, we hoped to sample most patterns of efficiencies that can be produced by a transient hexamer.
Parameters obtained by fitting the transient dimer model (Equation 26) to the pattern of efficiencies produced by the transient hexamer model are presented in Figure 3.2. The column containing panels A to M shows the predicted pairwise FRET efficiency, the column containing panels B to N shows the logarithm of the predicted equilibrium association constant and the column containing panels C to O shows the root-mean-square error of the fit normalised by the mean of the predicted FRET efficiencies (i.e. the coefficient of variation of the root-mean-square error of the fit). Values are presented through colours, as described by the colourbar above each column. Histograms of the values obtained from all 125 simulations are presented below each column. Pairwise FRET efficiencies predicted by the transient dimer model often are greater than 0.5, which was expected since the apparent FRET efficiency produced by a hexamer is higher than the apparent FRET efficiency produced by a dimer. Similarly, the logarithms of the equilibrium association constants often are greater than 3, which was expected since the formation of hexamers can produce a steeper increase in the apparent FRET efficiency as a function of expression level than the formation of dimers. Overall, the transient dimer model could fit the patterns of efficiencies produced by the transient hexamer model surprisingly well, with a normalised root-mean-square error being less than 0.05 in most cases. Thus, during the analysis of the experimentally-acquired FRET efficiencies, we used the transient dimer model to characterize a transiently-association oligomer of any size.

In Figure 3.2, the simulation that resulted in the highest normalised root-mean-square error is labelled with 1, while simulations that produced fits with the normalised root-mean-square error in the 80th, 60th and 40th percentiles are labelled with 2, 3 and 4, respectively. Fits produced using the data from simulations 1, 2, 3 and 4 are presented in Figure 3.3.
Keq 2

Keq 3 = 0.001

Keq 3 = 0.1

Keq 3 = 1

Keq 3 = 10

Keq 3 = 1000

Pairwise FRET efficiency

Log of the association constant

Normalised root–mean–square error

Number of occurrences

Pairwise FRET efficiency

Log of the association constant

Normalised root–mean–square error
Figure 3.2: The quality of the fits that are achieved by using the model for a transient dimer to fit FRET efficiencies produced using the model for a transient hexamer. FRET efficiencies were simulated at expression levels ranging from 0 to 26 A.U. using the model for a transient hexamer and the resulting patterns of efficiencies were fit using the model for a transient dimer. The acceptor-to-donor ratio was set to 10 and the pairwise FRET efficiency was set to 0.50 in each simulation. The equilibrium association constants for the formation of dimers ($K_{eq1}$) are shown on the x-axes, the equilibrium association constants for the formation of tetramers ($K_{eq2}$) and show in the y-axes and the equilibrium association constants for the formation of tetramers hexamers ($K_{eq3}$) are shown at the top of each row. The column containing panels A to M shows the pairwise FRET efficiency, the column containing panels B to N shows the log of the equilibrium association constant and the column containing panels C to O shows the root-mean-square error of the fit normalised by the mean of the predicted FRET efficiencies (i.e. the coefficient of variation of the root-mean-square error of the fit). Values are presented through colours, as described by the colourbar above each column. Histograms of the values obtained from all 125 simulations are presented below each column. Simulations labelled 1, 2, 3 and 4 correspond to Panels 1, 2, 3 and 4 in Figure 3.3.
Figure 3.3: Examples of the fits that are achieved by using the model for a transient dimer to fit FRET efficiencies produced using the model for a transient hexamer. Panel 1 shows the worst fit that was achieved out of the 125 simulations that were performed. 80% of simulations achieved a fit that is better than what is presented in Panel 2. 60% of simulations achieved a fit that is better than what is presented in Panel 3. 40% of the simulations achieved a fit that is better than what is presented in Panel 4. The quality of the fits was quantified using the coefficient of variation of the root-mean-square error of the fit, calculated by normalising the root-mean-square error by the average of the FRET efficiencies obtained for a given simulation. Parameters that were used for each simulation, and parameters that were obtained by fitting the simulated data to the transient dimer model, are presented at the top of each panel. Panels 1, 2, 3 and 4 correspond to the simulations labelled 1, 2, 3 and 4 in Figure 3.2.
3.3.6  Bystander FRET model

It is possible for the protein in question to be monomeric and to have no affinity for other, similar proteins. In that case the models described above would predict zero FRET between the donor- and acceptor-labelled molecules. However if the proteins are expressed at a very high level and are confined to small plasma membrane microdomains, then each donor could have one or more acceptors close enough for FRET to occur simply by chance, due to the close packing of membrane proteins. This situation can be described by Equation 30, which was derived in [79] and describes the efficiency of energy transfer between donors and randomly dispersed acceptors. Here $C_{\text{acceptor}}$ is the number of acceptor molecules per Förster radius squared ($R_0^2$), $\gamma$ is the lower incomplete gamma function as described by Equation 31, $\alpha$ is the distance of closest approach between a donor and an acceptor ($R_c$) normalised by the Förster radius ($R_0$) and raised to the sixth power (i.e., $\alpha = (R_c/R_0)^6$), and $\lambda$ is the ratio of the time after excitation over the lifetime of the fluorophore.

\[
E_{\text{bystander}} = 1 - \int_0^\infty \exp[-\lambda] \exp[-\pi C_{\text{acceptor}} \gamma(2/3, \lambda/\alpha) \lambda^{1/3}] \exp[\pi C_{\text{acceptor}} \alpha^{1/3} (1 - e^{-\lambda/\alpha})] d\lambda
\]  

\[
\gamma(x, y) = \int_0^y x^{-1} e^{-z} dz
\]  

Since in our experiments we calculated the concentration of donors and acceptors in arbitrary units ($[A]_t$), another parameter $k_{\text{corr}}$, representing the conversion factor from arbitrary units to molecules per $R_0^2$ ($C_{\text{acceptor}}$), had to be introduced into Equation 30. The resulting Equation 32 could be fit to the data using the lsqnonlin function in Matlab, with the indefinite integral evaluated using a 32-point Gauss-Laguerre procedure upon each iteration of the fitting algorithm [79].

\[
E_{\text{bystander}} = 1 - \int_0^\infty \exp[-\lambda] \exp[-\pi k_{\text{corr}} [A]_t \gamma(2/3, \lambda/\alpha) \lambda^{1/3}] \exp[\pi k_{\text{corr}} [A]_t \alpha^{1/3} (1 - e^{-\lambda/\alpha})] d\lambda
\]
3.3.7 Stable dimer with bystander FRET model

If proteins that form stable dimers and higher-order oligomers are confined to small microdomains at a high level of expression, in addition to FRET between stable dimers we could also observe bystander FRET between non-interacting protomers. Thus, as with monomers, the apparent FRET efficiency would increase as a function of expression level.

If the protein in question is a dimer, it will form acceptor-acceptor dimers, donor-donor dimers and donor-acceptor dimers, in various proportions depending on the acceptor to donor ratio. The acceptor-acceptor dimers will not be excited by the 405 nm laser, and will contribute to the apparent FRET efficiency only as acceptors for bystander FRET (Figure 3.4, panel A). The donor-donor dimers are not bound to an acceptor, and will contribute to the apparent FRET efficiency only as donors for bystander FRET (Figure 3.4, panel B). The donor-acceptor dimers will show an apparent FRET efficiency that will be a non-linear combination of bystander FRET between non-interacting protomers and pairwise FRET within the dimeric complex (Figure 3.4, panel C). This non-linear combination of FRET can be modelled as coming from a trimer with one donor and two acceptors, with the two acceptors being different distances away from the donor (Figure 3.4, panel D). In this model, the interface between the donor and the first acceptor corresponds to the pairwise FRET efficiency, and the interface between the donor and the second acceptor corresponds to bystander FRET.
| Dimeric form | 
| --- | --- | --- |
| ![](image1) | ![](image2) | ![](image3) |
| Fraction of donors that exist in this form | 0 | $1 - P_A$ | $P_A$ |
| Apparent FRET efficiency given by donors in this form | N/A | $E_{\text{bystander}}$ | $E_{\text{pairwise + bystander}}$ |

**Figure 3.4: Schematic showing the three possible combinations of donors and acceptors for a stable dimer with bystander FRET.** Acceptor-acceptor dimers will not be excited by the 405 nm laser (panel A) and will contribute to the apparent FRET efficiency only as acceptors for bystander FRET (thin, light-green arrows in panels B and C). Donor-donor dimers will only show bystander FRET (thin, light-green arrows in panel B). Donor-acceptor dimers show both bystander FRET (thin, light-green arrows in panel C) and intradimeric pairwise FRET (thick, light-green arrow in panel C). Donor-acceptor dimers can be modelled as trimers, with the first donor-acceptor interface corresponding to intradimeric FRET (thick, light-green arrow in panel D) and the second donor-acceptor interface corresponding to bystander FRET (thin, light-green arrows in panel D).
Under these simplifying assumptions, the total FRET efficiency \( E_{\text{stable dimer + bystander}} \) observed with various acceptor-to-donor ratios can be described by Equation 33.

\[
E_{\text{stable dimer + bystander}} = P_A \cdot E_{\text{pairwise + bystander}} + (1 - P_A) \cdot E_{\text{bystander}}
\]

To obtain a model for the combined effect of intradimeric and bystander FRET (\( E_{\text{pairwise + bystander}} \)) we can start with Equations 34, which describes the FRET efficiency of the \( i \)-th donor in an oligomeric complex with \( k \) donors and \( n-k \) acceptors different distances away from the donor [81]. In the case of our hypothetical trimer (Figure 3.4, panel D), we have one donor and two acceptors, so \( i = 1, k = 1, n = 3 \). \( j \) is the index of each acceptor, and in our model \( j = 1 \) corresponds to the acceptor for pairwise FRET, and \( j = 2 \) corresponds to the acceptor for bystander FRET. Since we are using fluorophores, the Förster radius is the same for each donor-acceptor pair and \( R_{0,ij,q}^0 = R_0 \). With these factors taken into account, Equation 34 can be reduced to Equation 35.

\[
E_{i,k,n} = \sum_{j=1}^{n-k} \frac{(R_{i,j}^0/r_{i,j})^6}{1 + \sum_{j=1}^{n-k} (R_{i,j}^0/r_{i,j})^6}
\]

\[
E_{\text{pairwise + bystander}} = \sum_{j=1}^{2} \frac{(R_j^0/r_j)^6}{1 + \sum_{j=1}^{2} (R_j^0/r_j)^6} = \left(\frac{R_0}{r_{\text{pairwise}}}\right)^6 + \left(\frac{R_0}{r_{\text{bystander}}}\right)^6
\]

The Förster equation (Equation 36) relates the distance \( r_j \) between one donor and one acceptor to the pairwise FRET efficiency \( E_j \). Rearranging the Förster equation to isolate the ratio of the Förster distance to the intermolecular distance \( (R_j^0/r_j)^6 \), we obtain Equation 37.

\[
E_j = \frac{(R_j^0/r_j)^6}{(R_0/r_j)^6 + 1}
\]
By substituting Equation 37 into Equation 35, we obtain Equation 38, which can be simplified to Equation 39. This equation describes the apparent FRET efficiency of a donor-acceptor dimer as a function of pairwise and bystander FRET efficiencies.

\[
E_{\text{pairwise + bystander}} = \frac{E_{\text{pairwise}} + E_{\text{bystander}}}{1 - E_{\text{pairwise}} + E_{\text{bystander}}} = \frac{E_{\text{pairwise}} - 2 \cdot E_{\text{pairwise}} \cdot E_{\text{bystander}} + E_{\text{bystander}}}{1 - E_{\text{pairwise}} \cdot E_{\text{bystander}}}
\]

Finally, by substituting Equation 39 into Equation 33, we obtain Equation 41, which describes the apparent FRET efficiency for a system of clustered dimers. This model is parameterised by \(E_{\text{pairwise}}\), which is a constant, and \(E_{\text{bystander}}\), which can be obtained using Equation 32, and depends on the level of acceptor expression and the degree of clustering.

\[
E_{\text{stable dimer + bystander}} = P_A \left( E_{\text{pairwise}} - 2 \cdot E_{\text{pairwise}} \cdot E_{\text{bystander}} + E_{\text{bystander}} \right) + \left( 1 - P_A \right) E_{\text{bystander}}
\]

It is useful to rewrite Equation 40 as Equation 41, since in most cases it was fit to apparent FRET efficiencies that were plotted as a function of the acceptor-to-donor ratio \([A]/[D]\) rather than the acceptor mole fraction \(P_A\).

\[
E_{\text{stable dimer + bystander}} = \left( \frac{1}{1 + \frac{1}{[A]/[D]}} \right) \left( E_{\text{pairwise}} - 2 \cdot E_{\text{pairwise}} \cdot E_{\text{bystander}} + E_{\text{bystander}} \right) + \left( 1 - \frac{1}{1 + \frac{1}{[A]/[D]}} \right) E_{\text{bystander}}
\]
3.4 Materials and methods

3.4.1 Constructs and vectors

Constructs containing the sequence for the M2 receptor inside a pcDNA3.1 vector, tagged with GFP2 or eYFP at either the N or C terminus, were described previously [60]. Constructs containing the M1 muscarinic receptor and the β1 and β2 adrenergic receptors were created by Luca Pisterzi using the same protocol. Luca Pisterzi also made the constructs containing the first 32 amino acids from the human Gαi1 protein, including the myristoylation palmitoylation sequence, followed by eYFP, GFP2, or eYFP and GFP2 fused in tandem. Those constructs are referred to as MP-GFP2, MP-eYFP and MP-eYFP-GPF2.

pcDNA3.1 vectors containing human CD28 and CD86, tagged with CFP at the C terminus, were a gift from Dr. Bünnemann [62]. CFP was replaced with eYFP or GFP2 using XbaI and NotI restriction sites and primers 1F and 1R (Table 2). In order to generate pSNAPf vectors containing CD28, CD86 or the M2 receptor, tagged either with eYFP or GFP2 at the N-terminus, sequences encoding those proteins were amplified through PCR using primers 2F and 2R, 3F and 3R, or 4F and 4R (Table 2), respectively, and were inserted into the pSNAPf vector following the α7 signal sequence and either eYFP or GFP2, using XhoI and BspEI restriction sites. The pSNAPf vector contains a neomycin resistance gene following an IRES promoter, which allowed for the easy creation of stable cell lines.

Constructs containing canine caveolin-1, tagged with dsRed at the C terminus, was a gift from Dr. Fairn [83]. Caveolin-1 was cloned into the pcDNA3.1 vectors containing eYFP and GFP2 downstream of the caveolin-1 insertion site using HindIII and BamHI restriction sites.

Constructs containing the human GABA\textsubscript{B1} and GABA\textsubscript{B2} receptors were a gift from Dr. Salahpour. The coiled-coil domains from these receptors were amplified using primers 5F and 5R for the GABA\textsubscript{B1} receptor and primers 6F and 6R for and GABA\textsubscript{B2} receptor, and were inserted immediately following the M2 receptor using restriction sites BspEI and NotI.
Palmitoylation-deficient mutants of the M2 receptor were created by substituting the cysteine at position 457 with an alanine. This was done using standard PCR techniques, primers 4F and 7R, and a pSNAPf vector containing an α7 signal sequence, either eYFP or GFP2, and the M2 receptor. The α7 signal sequence is a cleavable endoplasmic reticulum targeting sequence, which has been shown previously to improve membrane localization of transmembrane proteins [84]. All fluorophores used in this study contained the A206K mutation which eliminated their propensity to dimerise [73]. All sequences were confirmed by DNA sequencing.

A ribbon diagram for each construct that was used in the FRET study is presented in Figure 3.5.

Table 2: List of all the primers that were used to generate DNA constructs for the Förster resonance energy transfer study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F</td>
<td>GAATTCTCTAGAAAGGGCGAGGAGCTGTTCAGACCTGTTCA</td>
</tr>
<tr>
<td>1R</td>
<td>CATGGACGAGCTTGTACAAGTCCGGATAAGCGGCGAGCAGGATTC</td>
</tr>
<tr>
<td>2F</td>
<td>CCCCCTTCAGAGTCTTGTAGCGTACGACAATG (5Xho-CD28)</td>
</tr>
<tr>
<td>2R</td>
<td>GAATTCGCGGCCGCTTATCCGGACCTGCTCCTTACTCTCCTACC</td>
</tr>
<tr>
<td>3F</td>
<td>CCCCCTTCAGAGTCTTGTAGCGTACGACAATG (5Xho-CD86)</td>
</tr>
<tr>
<td>3R</td>
<td>GAATTCGCGGCCGCTTATCCGGACCTGCTCCTTACTCTCCTACC</td>
</tr>
<tr>
<td>4F</td>
<td>GCCCCCTTCAGAGTCTTGTAGCGTACGACAATG (5Xho-M2)</td>
</tr>
<tr>
<td>4R</td>
<td>GAATTCGCGGCCGCTTATCCGGACCTGCTCCTTACTCTCCTACC</td>
</tr>
<tr>
<td>5F</td>
<td>CCCCCCTTCAGAGTCTTGTAGCGTACGACAATG (GABBR1 forward)</td>
</tr>
<tr>
<td>5R</td>
<td>GGGGGCGGCCGCTTATCCGGACCTGCTCCTTACTCTCCTACC (GABBR1 reverse)</td>
</tr>
<tr>
<td>6F</td>
<td>CCCCCCTTCAGAGTCTTGTAGCGTACGACAATG (GABBR2 forward)</td>
</tr>
<tr>
<td>6R</td>
<td>GGGGGCGGCCGCTTATCCGGACCTGCTCCTTACTCTCCTACC (GABBR2 reverse)</td>
</tr>
<tr>
<td>7R</td>
<td>GGGGGCGGCCGCTTATCCGGACCTTGTAGCGCCTATGTTCTTATAATGAGCCATGAGAAGG (M2 noPalm)</td>
</tr>
</tbody>
</table>
Figure 3.5: Ribbon diagrams showing all of the constructs that were used in the FRET study. α7 is a cleavable endoplasmic reticulum localisation sequence from the α7 nicotinic acetylcholine receptor. M2 is the M2 muscarinic receptor. C457A is a cysteine to alanine mutation that blocks the palmitoylation of the M2 muscarinic receptor. R1 and R2 are the coiled-coil domains from the GABA<sub>B1</sub> and GABA<sub>B2</sub> receptors, respectively. M1 is the M1 muscarinic receptor. ADRB2 is the β2 adrenergic receptor. CD28 is a dimeric integral membrane protein. CD86 is a monomeric integral membrane protein. Cav1 is the caveolin-1 scaffolding protein. Ga11 is the alpha subunit of a heterotrimeric G protein. MP is a myristoylation palmitoylation tag obtained from the first 32 amino acids of Ga11.
3.4.2 Cell culture

Chinese hamster ovary (CHO) cells were grown in DMEM media supplemented with 5% fetal bovine serum, 0.5% non-essential amino acids and 0.5% w/w Penicillin-Streptomycin (Invitrogen). Cells were grown at 37 °C in a humidified atmosphere with 5% CO₂. For imaging, cells were seeded on number 1 coverslips 10 mm in diameter (Fisher Scientific) inside six-well plates at a density of 1x10⁶ cells per well. Cells were transfected with plasmid DNA at an acceptor to donor plasmid ratio ranging from 3:1 to 1:20 using polyethylenimine (Polyscience Inc.), and were incubated in phenol red- and FBS-free media for 16 to 48 hours prior to imaging.

3.4.3 Imaging

Spectral images were acquired at the Mount Sinai imaging facility in Toronto, using the Nikon C1si microscope and a 60x oil immersion lens. The diameter of the pinhole was set to 66 µm, and the pixel dwell time was 4 µs. The power output of the 405 nm and 514 nm lasers was measured at the objective after each imaging session, and ranged from 13.90 to 30.60 µW for the 405 nm laser, and from 1.70 to 2.75 µW for the 514 nm laser.

Two spectral images, using 405 nm and 514 nm excitation, were acquired for each cell. The 405 nm laser excited only the GFP2 fluorophores, and so the first spectral image contained emission both from GFP2 fluorophores that were excited directly by the laser and eYFP fluorophores that were excited indirectly through FRET. The 514 laser excited only eYFP fluorophores, and the second spectral image contained emission only from eYFP. Each spectral image contained 32 channels, covering the emission spectrum from 470 nm to 630 nm, in 5 nm intervals.

3.4.4 Automated cell cropping

Spectral images of cells were imported into Matlab using the Bioformats importer [85]. We used the mean intensity of the first channel as an estimate of the background intensity and subtracted
that value from every channel in the spectral image. This could be done because both GFP2 and eYFP have negligible emission at 470 nm, which is the wavelength of the first channel.

In order to crop individual cells, we first averaged each channel in the image using a 25 pixel by 25 pixel rolling-average filter. For images that were acquired using 405 nm excitation, we unmixed the spectrum from each pixel into the GFP2 and eYFP components, using reference spectra that were acquired from cells expressing only GFP2 or eYFP. For images that were acquired using 514 nm excitation, we fit the obtained spectrum using only the eYFP reference spectrum. In both cases, we compared the goodness of fit of the reference spectra to the observed single-pixel emission spectra using the $R^2$ value, and we created a binary image, referred to as the cropping mask, that kept only the pixels with an $R^2$ value greater than 0.8 for both images. This cropping mask was smoothed and expanded using the Matlab bwareaopen function, and it was segmented into disjoint components using the Matlab bwconncomp function. In most cases, each disjoint component would correspond to an individual cell. However, when two or more cells in an image were in contact or in close proximity, they would be segmented into a single component.

The cropping mask for each disjoint component then was applied to the raw, non-averaged spectral images, producing the average emission spectra for the cell covered by that cropping mask. Those cell-averaged emission spectra again were unmixed into GFP2 and eYFP components for the 405 nm excited image, and into the eYFP component for the 514 nm excited image. By following the procedures outlined in Section 3.3.2, we were able to calculate the FRET efficiency and the amount of donor and acceptor present in that cell.

3.4.5 Data analysis

All data analysis was performed using Matlab (R2012a, The MathWorks, Natick, Massachusetts). Equations were fit to the data using the lsqnonlin solver in the optimisation toolbox.
3.5 Results

3.5.1 Presentation of the data

CHO or HEK293 cells were transfected with different combinations of GFP2- and eYFP-tagged proteins, and FRET efficiencies of individual cells were measured using a spectrally-resolved microscope. Figure 3.6 to Figure 3.10 in the results section and Figure A.1 to Figure A.14 in the appendix show sample images (panels A, B and C) and apparent FRET efficiencies (panels D to K) of cells expressing a given protein pair. In panels A, B and C, the green channel corresponds to the emission from GFP2-tagged proteins (donors) and the red channel corresponds to the emission from eYFP-tagged proteins (acceptors). In panels D to K, each point corresponds to a single cell, and different colours indicate cells that were transfected with a different acceptor-to-donor ratio or were imaged on different days. Apparent FRET efficiencies of individual cells are plotted either as a function of acceptor expression (panels D to G) or as a function of acceptor-to-donor ratio (panels H to K). Panel L shows acceptor expression level plotted as a function of acceptor-to-donor ratio, with the correlation coefficient and the slope and y-intercept of the line of best fit (dashed line) presented at the top of the panel.

Apparent FRET efficiencies are fit by models for bystander FRET (Equation 32, panels D and H), a stable dimer (Equation 19, panels E and I), a stable dimer with bystander FRET (Equation 41, panels F and J) and a transient dimer (Equation 26, panels G and K). The parameters obtained from those fits, as well as the coefficient of variation of the root-mean-square error of the fit (CV(rmse)), are presented at the top of the corresponding panels. The model for bystander FRET does not depend on the acceptor-to-donor ratio, and panel D shows uncorrected FRET efficiencies plotted as a function of acceptor expression. Similarly, the model for a stable dimer does not depend on acceptor expression level, and panel I shows uncorrected FRET efficiencies plotted as a function of acceptor-to-donor ratio. FRET efficiencies presented in panels H and E were corrected to an acceptor expression level of 0.005 or an acceptor-to-donor ratio of 10 using Equation 42 and Equation 43, respectively. In those equations, $f_{\text{bystander}}(x, [A], p)$ corresponds to
the bystander FRET model (Equation 32), and $f_{\text{dimer}}(x, [A]_i/[D]_i, p)$ corresponds to the stable dimer model (Equation 19), with $p$ being the parameters obtained from the least-squares fit of the model to the data.

$$x_{[A]_i, 0.005} = x \cdot \frac{f_{\text{bystander}}(x, 0.005, p)}{f_{\text{bystander}}(x, [A]_i, p)}$$

$$x_{[D]_i, 10} = x \cdot \frac{f_{\text{dimer}}(x, 10, p)}{f_{\text{dimer}}(x, [A]_i/[D]_i, p)}$$

The model for a transient dimer and the model for a stable dimer with bystander FRET are functions of both the acceptor expression level and the acceptor-to-donor ratio, and to present the fits to the two models on two-dimensional coordinates, FRET efficiencies in panels F and G were corrected to a constant acceptor-to-donor ratio of 10 using Equation 44, and FRET efficiencies in panels J and K were corrected to a constant acceptor expression level of 0.005 using Equation 45 [86]. In Equation 44 and Equation 45, $f(x, [A]_i/[D]_i, [A]_i, p)$ corresponds either to the transient dimer model (Equation 26) or to the stable dimer with bystander FRET model (Equation 41), and $p$ corresponds to the parameters obtained from the least-squares fit of the corresponding model to the data.

$$x_{[D]_i, 10} = x \cdot \frac{f(x, 10, [A]_i, p)}{f(x, [A]_i/[D]_i, [A]_i, p)}$$

$$x_{[A]_i, 0.005} = x \cdot \frac{f(x, [A]_i/[D]_i, 0.005, p)}{f(x, [A]_i/[D]_i, [A]_i, p)}$$

### 3.5.2 Fits achieved using different models

The first protein we examined was our monomeric control CD86. FRET efficiencies for cells co-expressing GFP2-CD86 and eYFP-CD86 range from 0% at low levels of expression to over 40%
at high levels of expression (Figure 3.6, panel D). The pattern of efficiencies is fit well by models for bystander FRET (Figure 3.6, panels D and H; CV(rmse) = 0.14), a stable dimer with bystander FRET (Figure 3.6, panels F and J; CV(rmse) = 0.14) and a transient dimer (Figure 3.6, panels G and K; CV(rmse) = 0.14). The pattern of efficiencies is described poorly by the model for a stable dimer (Figure 3.6, panels E and I; CV(rmse) = 0.26). Apparent FRET efficiencies transformed to a constant acceptor expression level of 0.005 using the optimal fit of the bystander FRET model appear largely independent of the acceptor-to-donor ratio (Figure 3.6, panel H). In contrast, apparent FRET efficiencies transformed to a constant acceptor-to-donor ratio of 10 using the optimal fit of the stable dimer model maintain their dependence on the acceptor expression level (Figure 3.6, panel E). This shows that the variance in the data can be captured by the bystander FRET model but not by the stable dimer model. When the transient dimer model is fit to the data, the estimated equilibrium association constant is relatively small (Figure 3.6, panels G and K; Keq = 71), which suggests that GFP2- and eYFP-tagged proteins have a low affinity for each other. When the stable dimer with bystander FRET model is fit to the data, the estimated pairwise FRET efficiency also is small (Figure 3.6, panel H; Ep = 0.037), which suggests that very few oligomers are formed. Taken together, the fits obtained using the four models suggest that the efficiencies obtained for CD86 are a result of bystander FRET or transient oligomerisation, and are not due to the stable oligomerisation, which is consistent with our use of CD86 as a monomeric control.

FRET efficiencies obtained for the M2 receptor tagged with fluorophores either at the cytosolic C terminus (Figure 3.7) or the extracellular N terminus (Figure A.1) are similar to those obtained for the monomeric control CD86, and the same conclusions can be made about the quality of the fits achieved using the four models. The bystander FRET model fits the data better than the stable dimer model (CV(rmse) = 0.21 vs. CV(rmse) = 0.38), the pairwise FRET efficiency predicted by the stable dimer with bystander FRET model is low (Figure 3.7, panels F and J, Ep = 0.052) and the equilibrium association constant predicted by the transient dimer model also is low (Figure 3.7, panels G and K, Keq = 86). Furthermore, the pattern of efficiencies observed between different M2 receptors is similar to the pattern observed between M2 muscarinic receptors and M1 muscarinic receptors (Figure A.2) or β2 adrenergic receptors (Figure A.3).
Thus, the efficiencies obtained for the M2 muscarinic receptor, like the efficiencies obtained for CD86, appear to be the result of bystander FRET or transient oligomerisation and are not due to the specific and constitutive oligomerisation of the receptor.

As a transient oligomer control, we co-expressed GFP2-tagged Gαi1 G protein subunits with eYFP-tagged M2 receptors, either in the absence (Figure 3.8) or presence (Figure A.8) of Gβγ. While the stability of the receptor-G protein complex still is in question, there is general consensus that GPCRs facilitate the exchange of GDP to GTP at the Ga subunit through direct interactions [22] and that those interactions are not permanent [16,19]. Thus we expected that the pattern of efficiencies obtained for cells co-expressing GFP2-tagged Gαi1 and eYFP-tagged M2 receptor would be distinctly different from the pattern of efficiencies obtained for the monomeric control CD86, and would be fit better by the model for a transient dimer than any other model. In contrast to this prediction, the pattern of efficiencies observed for cells co-expressing GFP2-Gαi1 and eYFP-M2 (Figure A.4) is similar to the pattern observed between CD86 molecules (Figure 3.6) and between different M2 receptors (Figure 3.7). The efficiencies are described best by the model for a transient dimer (Figure 3.8, panels G and K), but the difference in the quality of the fits is not great and is comparable to the difference observed for other proteins (e.g. CD28 in Figure 3.10). This suggests either that the affinity of the receptor for the G protein is low or that the amount of background FRET is too high to be able to distinguish a monomer from a transient dimer.

In order to create a dimeric control with the same hydrodynamic radius as the M2 muscarinic receptor, we attached the coiled-coil domains from the GABA_B1 and GABA_B2 receptors to the C termini of the GFP2- and eYFP-tagged M2 receptors. The coiled-coil domains promote and stabilise the dimeric form of the receptor and have been shown previously to bestow the same pattern of efficiencies on the protein to which they are fused as is observed for the wildtype GABA_B receptor dimer [76]. When M2 receptors with the coiled-coil domains (GFP2-M2R1 and eYFP-M2R2) were expressed in CHO cells, most of GFP2-M2R1 was retained in the endoplasmic reticulum (Figure A.10, panels A, B and C), which made the data difficult to interpret. When M2 receptors with the coiled-coil domains were expressed in HEK293 cells,
however, GFP2-tagged and eYFP-tagged receptors were more co-localised (Figure 3.9, panels A to C), and the observed pattern of efficiencies is consistent with our expectation for a stable dimer. FRET efficiencies are described better by a model for a stable dimer than the model for bystander FRET (CV(rmse) = 0.16 vs. CV(rmse) = 0.32), and while the models for a stable dimer with bystander FRET and for a transient dimer are able to achieve even better fits to the data, the pairwise FRET efficiency predicted by the stable dimer with bystander FRET model is high (Figure 3.9, panels F and J, Ep = 0.21) and is comparable to the pairwise FRET efficiency predicted by the stable dimer model (Figure 3.9, panels E and I, Ep = 0.27), and the equilibrium association constant predicted by the transient dimer model also is high (Figure 3.9, panels G and K, Keq = 1.5×10^3) and shows that the dimers are formed even at low levels of expression.

FRET efficiencies obtained for the dimeric control CD28 (Figure 3.10) follow a distinctly different pattern from what is observed for the proteins discussed above. The model for a stable dimer (Figure 3.10, panels E and I; CV(rmse) = 0.14) fits the data better than the model for bystander FRET (Figure 3.10, panels D and H; CV(rmse) = 0.24). While the quality of the fits obtained using the stable dimer with bystander FRET model (Figure 3.10, panels F and J; CV(rmse) = 0.14) and the transient dimer model (Figure 3.10, panels G and K; CV(rmse) = 0.14) are comparable to the quality of the fit obtained using the stable dimer model, the pairwise FRET efficiency obtained using the stable dimer with bystander FRET model is high (Figure 3.10, panels F and J, Ep = 0.59) and is a reasonable estimate of the pairwise FRET efficiency for a CD28 dimer. The association constant obtained using the transient dimer model also is exceedingly high (Figure 3.10, panels G and K, Keq = 1.3×10^4) and implies that oligomers form even at low levels of expression. Thus, the data for the dimeric control CD28 shows that the pattern of efficiencies obtained for a stable covalently-linked dimer is distinctly different from what is obtained for a monomer CD86 and for other transiently or non-specifically interacting proteins, even if the magnitude of the efficiencies is comparable.
Figure 3.6: FRET efficiencies obtained for CHO cells co-expressing CD86-GFP2 and CD86-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure 3.7: FRET efficiencies obtained for CHO cells co-expressing M2-GFP2 and M2-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure 3.8: FRET efficiencies obtained for CHO cells co-expressing Gαi1-GFP2 and M2-eYFP, in the absence of Gβγ. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
GFP2-M2R1, eYFP-M2R2 (in HEK293 cells)

Figure 3.9: FRET efficiencies obtained for HEK293 cells co-expressing GFP2-M2R1 and eYFP-M2R2. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure 3.10: FRET efficiencies obtained for CHO cells co-expressing CD28-GFP2 and CD28-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
In the figures above (Figure 3.6 to Figure 3.10):
Panels A - C show sample images of cells co-expressing the indicated protein pair, with the
green channel corresponding to emission from the GFP2-tagged protein and the red channel
 corresponding to emission from the eYFP-tagged protein. Panels D to K all show the same FRET
efficiencies obtained from the same cells, with each dot representing a single cell. Dots that have
the same colour represent cells that were imaged from the same coverslip and were transfected
for the same period of time. Efficiencies were fit by models for bystander FRET (panels D, H), a
stable dimer (panels E, I), a stable dimer with bystander FRET (panels F, J) and a transient dimer
(panels G, K). Efficiencies presented in panels D to G were transformed to a constant acceptor-
to-donor ratio of 10, and efficiencies presented in panels H to K were transformed to a constant
acceptor expression level of 0.005, using the corresponding model (Equations 42 - 45). The blue
lines show the optimal fit achieved by those models, and the values at the top of panels D, E, F
and G show the optimal parameters for each model and the root-mean-square error of the fit.
Panel L shows acceptor expression level plotted as a function of acceptor-to-donor ratio. The
dashed line is the line of best fit through the data. The slope and y-intercept of the dashed line is
given at the top of the panel, along with the correlation coefficient relating acceptor expression
level and acceptor-to-donor ratio.
3.5.3 Separation of protein pairs into clusters based on the fits achieved using the four models

In the sections above, we decided whether or not the protein pair in question forms a stable complex mainly by looking at the quality of the fits that are achieved using the bystander FRET model and the stable dimer model and by looking at the equilibrium association constant predicted by the transient dimer model. In order to create an objective computational approach that would allow us to separate a large number of different protein pairs into clusters based on their propensity to form a stable complex, we generalised the above criteria into two numerical measures, and used those measures to plot all protein pairs that were investigated (Figure 3.11) on the same axes (Figure 3.12).

First, we looked at the difference in the quality of the fits that are achieved using the bystander FRET model and the stable dimer model, as indicated by the difference in the coefficient of variation of the root-mean-square error (i.e. CV(rmse) bystander FRET model – CV(rmse) stable dimer model). This difference in CV(rmse) is plotted on the x axis in Figure 3.12. The pattern of efficiencies produced by an oligomer should be fit better by the stable dimer model and should give a positive difference in the CV(rmse). Accordingly, the difference in CV(rmse) is greater than zero for MP-eYFP-GFP2, caveolin-1, CD28 and the M2 fused with the GABA<sub>B</sub> coiled coil domains and expressed in HEK293 cells (Figure 3.12, x axis > 0). The pattern of efficiencies produced by a monomer should be fit better by the bystander FRET model and give a negative difference in the CV(rmse). Accordingly, the difference in CV(rmse) is less than zero for the monomeric control CD86 and for all other protein pairs that were investigated, including the wildtype M<sub>2</sub> muscarinic receptor (Figure 3.12, x axis < 0).

Next, we looked at the logarithm of the equilibrium association constant predicted by the transient dimer model (i.e. log(Keq)). Monomers should have a low affinity towards each other and should show a small equilibrium association constant, while oligomers should have a high affinity towards each other and should show a high equilibrium association constant. The log(Keq) variable is plotted on the y-axis in Figure 3.12, and, as expected, the monomeric control CD86 has a low log(Keq) value of less than 2, the dimeric control CD28 has a high
log(Keq) value that is greater than 4 and the oligomeric control caveolin-1 has an even higher log(Keq) value that is greater than 6. The M₂ receptor with the GABA₆ coiled coil domains expressed in HEK293 cells has a log(Keq) value that is greater than 3 and is distinctly separated from the monomeric control CD86 and all other protein pairs whose oligomeric size is in question.

If any of the constants that are used to calculate the acceptor-to-donor ratio are not accurate, the calculated acceptor-to-donor ratio will be off by some multiple for all cells that are imaged. This, in turn, may lead to a poor fit by the stable dimer model, even for proteins that exist as stable oligomers. To account for this possibility, an extra variable $c$ was introduced into Equation 19 (Equation 21) and was allowed to vary between 0.2 and 5, which corresponds to a 5-fold underestimate or overestimate, respectively, of the acceptor-to-donor ratio. Nevertheless, most protein pairs that are fit worse by the stable dimer model than the bystander FRET model (Figure 3.12, x axis < 0) also are fit worse by the stable dimer with a correction factor model than the bystander FRET model (Figure 3.13, x axis < 0). The only exception is “M₂-GFP2 CD86-eYFP”, which was fit better by the stable dimer model with a correction factor of 0.2 than by the bystander FRET model. However, since the stable dimer model was derived using the assumption that the number of donor-donor dimers, donor-acceptor dimers and acceptor-acceptor dimers follows the binomial distribution, it may not be an accurate model to describe the pattern of efficiencies between two entirely different proteins.
Figure 3.11: A schematic showing all proteins that were used in the Förster resonance energy transfer study. The M2 muscarinic receptor is a prototypical class A GPCR, and its oligomeric size was being investigated. M2 receptors with the C457A mutation cannot be palmitoylated, and they were used to test the effect of the palmitoyl group on the oligomeric size of the receptor. M1 muscarinic receptors and β2 adrenergic receptors are class A GPCRs, and they were coexpressed with M2 receptors as additional negative controls. M2 receptors signal by directly interacting with the Gα subunit of the heterotrimeric G protein, and M2 receptors were coexpressed with Gαi as a transient oligomer control. CD28 is an integral membrane protein that forms disulphide-linked dimers, and it was used as a dimeric control. GFP2-eYFP linked in tandem and targeted to the membrane with a myristoylation palmitoylation tag was used as a dimeric control with a constant one-to-one acceptor-to-donor ratio. Caveolin-1 is a membrane protein responsible for invaginations in the membrane called caveoli, and it was used as a higher-order oligomeric control. M2 receptors fused with the coiled-coil domains from the GABAB1 and GABAB2 receptors, which facilitate and stabilise receptor dimers, were used as dimeric controls with the same hydrodynamic radius as wildtype M2 receptors. All proteins used in this study were fused either to GFP2 or eYFP. The fluorophore is used as a prefix in the name if was fused to the N terminus of a protein (e.g. GFP2-M2 and eYFP-M2), and it is used as a suffix in the name if it was fused to the C terminus of a protein (e.g. M2-GFP2 and M2-eYFP). The only exceptions are Gαi1-GFP2 and Gαi1-eYFP, where the fluorophores were inserted at position 91 of the Gαi1 G protein subunit.
Figure 3.12: Clustering of different donor-acceptor pairs based on the fits that are achieved using the bystander FRET model, the transient dimer model and the stable dimer model. The x-axis shows the difference between the CV(rmse) achieved by the bystander FRET model and the CV(rmse) achieved by the stable dimer model. CV(rmse) is the coefficient of variation of the root-mean-square error of the fit, and indicates how well a particular model describes the data. A low CV(rmse) value indicates a good fit, and so points to the left of the red vertical dashed line are fit better by the bystander FRET model and points to the right of the red vertical dashed line are fit better by the stable dimer model. The y-axis shows the logarithm of the equilibrium association constant predicted by the transient dimer model \( (i.e. \log(Keq)) \). At a given concentration, proteins with a low Keq value are expected to form fewer oligomers than proteins with a high Keq value.
Figure 3.13: Clustering of different donor-acceptor pairs based on the fits that were achieved using the bystander FRET model, the transient dimer model and the stable dimer with a correction factor model. The x-axis shows the difference between the CV(rmse) achieved by the bystander FRET model and the CV(rmse) achieved by the stable dimer with a correction factor model. The correction factor was allowed to vary between 0.2 and 5, corresponding to a 5-fold underestimate or overestimate in the acceptor-to-donor ratio, respectively. CV(rmse) is the coefficient of variation of the root-mean-square error of the fit, and indicates how well a particular model describes the data. A low CV(rmse) value indicates a good fit, and so points to the left of the red vertical dashed line are fit better by the bystander FRET model and points to the right of the red vertical dashed line are fit better by the stable dimer with a correction factor model. The y-axis shows the logarithm of the equilibrium association constant predicted by the transient dimer model (i.e. log(Keq)). At a given concentration, proteins with a low Keq value are expected to form fewer oligomers than proteins with a high Keq value.
3.6 Discussion

3.6.1 Correlation between acceptor expression level and acceptor-to-donor ratio

For many of the GFP2- and eYFP-tagged protein pairs that were investigated, the acceptor expression level was highly correlated with the acceptor-to-donor ratio (panel L in Figure 3.6 to Figure 3.10 and Figure A.1 to Figure A.11). This close correlation was not expected, especially at high acceptor-to-donor ratios, as the total amount plasmid transfected always was the same. For example, at a 5:1 acceptor-to-donor ratio, the total amount of acceptor in the cell should equal to \( \frac{5}{1+5} = 83.3\% \) of all tagged proteins. If this ratio is increased to 40:1, the total amount of acceptors should be \( \frac{40}{1+40} = 97.6\% \), which corresponds to an increase of only 1.17 times, given that the total concentration of donors and acceptors stays the same. However we observed an approximately linear relationship between the acceptor expression level and the acceptor-to-donor ratio, and going from a 5:1 to a 40:1 acceptor-to-donor ratio often is accompanied by a comparable increase in acceptor expression (e.g. panel L in Figure 3.6, Figure 3.7 and Figure 3.8).

A possible explanation for this could be a non-linear relationship between the number of plasmids that enter the cell and the number of proteins that are expressed. For instance, the expression of GFP2- and eYFP-tagged proteins could depend on the concentration of the respective plasmid raised to some exponent. This exponent could be higher in cells that express the protein at a high level than in cells that express the protein at a lower level. At high acceptor-to-donor ratios, this would lead to a correlation between the number of eYFP molecules expressed and the acceptor-to-donor ratio. Salahpour et al. show that the acceptor-to-donor ratio increases as the cells are transfected for a longer period of time, which correlates with an increase in BRET [78]. It is possible that a similar effect is observed for cells transfected for the same period of time but that express proteins at different levels.
A low correlation between acceptor expression and acceptor-to-donor ratio was observed for CD28 (Figure 3.10, panel L, correlation coefficient = 0.26). However, this is not the reason why the FRET efficiencies for CD28 are fit well by the model for a stable dimer. Data obtained for the M2 receptor with the coiled coil domains from the GABA\textsubscript{B} receptors expressed in HEK cells also are fit well by the model for a stable dimer but show a high degree of correlation between acceptor expression level and acceptor-to-donor ratio (Figure 3.9, panel L, correlation coefficient = 0.84).

### 3.6.2 Comparison with previous FRET studies

In a previous study, Pisterzi \textit{et al.} [60] measured FRET efficiencies for CHO cells expressing the M2 muscarinic receptor tagged with eGFP or eYFP at the N terminus, and they conclude that the receptors form trimers or tetramers in live cells. Efficiencies obtained by Pisterzi \textit{et al.} through donor dequenching are comparable with the efficiencies that we report in this study, and range from 0 to 0.4 at acceptor-to-donor ratios of 0 to 20. When Pisterzi \textit{et al.} fit uncorrected FRET efficiencies using the model for a stable dimer, they obtain a pairwise FRET efficiency of 0.365, and when they fit FRET efficiencies that were corrected for donor photobleaching, they obtain a pairwise FRET efficiency of 0.465. We obtain a pairwise FRET efficiency of 0.23 when we use the same model to fit our data for the M2 muscarinic receptor tagged with fluorophores at the N terminus, but in our case it is clear that the model does not fit the data (Figure A.1, panels E and I).

Pisterzi \textit{et al.} conclude that M2 receptors form trimers or tetramers because the pairwise FRET efficiencies that they obtain by fitting the donor dequenching ($E_{d}^{\text{app}}$) and acceptor sensitised emission ($E_{a}^{\text{sce}}$) data to models for a trimer and a tetramer are in close agreement with the pairwise FRET efficiency of 0.20 to 0.24 that they obtain through fluorescent lifetime imaging (FLIM). However, this conclusion relies on the assumptions that there is no bystander FRET and no monomers present in the system, and those assumptions may not be correct. The authors rule out the presence of bystander FRET because they do not observe FRET between the M2 receptor
and the truncated variant of the frizzled-1 receptor, which were used as a monomeric control. However, in our experience, the truncated variant of the frizzled-1 receptor is retained in the endoplasmic reticulum and is not colocalised with the M2 receptor (Figure A.15). The authors suggest that monomers are largely absent from their system because when they fit $E_{\text{app}}^{ddq}$ and $E_{\text{app}}^{ase}$ efficiencies simultaneously using the model for a stable tetramer with a subpopulation of monomers, the inclusion of monomers leads to an increase in the sum of squares of the fit. However, they do not talk about the quality of the fits that are achieved using the model for a stable dimer, trimer or hexamer in the presence of monomers. The model for a stable dimer in the presence of monomers can produce a pattern of efficiencies that is identical to the pattern produced by the model for a stable dimer in the absence of monomers (Equations 20 and 19, respectively). The model for a stable hexamer in the presence of monomers can produce a pairwise FRET efficiency that coincides with the pairwise FRET efficiency of 0.20 to 0.24 that was obtained using FLIM measurements.

FRET efficiencies reported by Pisterzi et al. were acquired from transiently-transfected cells showing a high level of expression. While the authors conclude that M2 receptors exist as trimers or tetramers, they note that their data also is consistent with a scheme where receptors exist in equilibrium between monomers, dimers and higher-order oligomers, if that equilibrium is shifted towards oligomers in the cells that were imaged. In our case, the pattern of efficiencies that is obtained for the M2 receptor is nearly identical to the pattern that is obtained for the monomeric control CD86, and this suggests that M2 receptors do not exist as constitutive oligomers in live cells. While our data for the M2 muscarinic receptor cannot be described by the model for a stable dimer, if the cells that we imaged showed a higher level of expression with less variability, it is possible that we too would have achieved an adequate fit using that model. It appears, therefore, that the most consistent interpretation of the data presented by Pisterzi et al. and the data that we present in our work is that M2 receptors exist in equilibrium between monomers and oligomers and that the equilibrium is shifted towards oligomers at higher levels of expression.

Another FRET-based study measuring the oligomeric size of the β2 adrenergic receptor was published during the preparation of this manuscript [88]. Kawono et al. obtained FRET
efficiencies from live cells expressing β2 adrenergic receptors as well as monomeric, dimeric and tetrameric controls, all of which were labelled with synthetic Alexa dyes using coiled-coil tag-probes. The authors used a spectrally resolved microscope to measure FRET efficiencies, but, in contrast to our own work, they calculated apparent FRET efficiencies through acceptor sensitised emission and not through donor dequenching, and they fit those efficiencies using the model derived by Veatch and Stryer [80], which does not take into account FRET through quasi-parallel processes [81]. Kawono et al. observe negligible FRET between β2 adrenergic receptors under various conditions, and conclude that the receptors do not form constitutive oligomers in live cells. This coincides with the conclusion made by James et al. and is consistent with our data for the M2 muscarinic receptor at lower levels of expression. However Kawono et al. do not address the shortcomings of the James et al. study [77,78] and do not offer a way of separating specific and non-specific signal at high levels of expression. While coiled-coil tag-probes allow for better control of the acceptor-to-donor ratio across different treatments, they can be used only to label extracellular or transmembrane proteins and may suffer from non-specific or incomplete labelling of the protein of interest.

Many other studies that used FRET to characterise the oligomeric size of class A GPCRs have concluded that those GPCRs form oligomers [54-58,74]. Some of the limitations of those studies were discussed by James et al [76]. Most of the early FRET studies did not use proper monomeric and oligomeric controls and did not look at the change in apparent FRET efficiencies as a function of acceptor expression level. In some cases, the possibility of transient oligomerisation was not considered and the results were interpreted in terms of a strict dichotomy between monomers and constitutive oligomers.

3.6.3 The oligomeric state of class A GPCRs

The pattern of FRET efficiencies obtained for the M2 receptor is similar to the pattern obtained for the monomeric control CD86 and is distinctly different from the patterns obtained for oligomeric controls CD28 and caveolin-1. Furthermore, the pattern of efficiencies observed for
different M2 receptors is similar to the pattern observed between M2 receptors and M1 receptors or β2 receptors. This is consistent with the results reported by James et al. and suggests that M2 muscarinic receptors, like the β2 adrenergic receptors, do not form constitutive oligomers in live cells. However, unlike James et al., we are not persuaded that the FRET data “challenge the notion that [GPCRs] are innately predisposed to forming homo- or hetero-oligomers” [76]. The acceptor expression level and the acceptor-to-donor ratio often are highly-correlated, which makes the independent variable in cell-averaged FRET experiments poorly defined. Furthermore, it is difficult to distinguish between a monomer and a transient oligomer, and the patterns of efficiencies that we observe between monomeric CD86 molecules and between different M2 receptors are similar to the pattern that we observe between M2 receptors and G proteins, which are known to interact.

Several groups have reported that class A GPCR oligomers are unstable and that the formation of oligomers depends on the level of expression and the membrane microenvironment. Botelho et al. showed that the extent to which rhodopsin molecules form oligomers in reconstituted membranes depends on the protein-to-lipid ratio and on the thickness of the lipid bilayer [89]. A low protein-to-lipid ratio and a lipid bilayer 20-22 acyl carbons in width would favour the formation of monomers, while a high protein-to-lipid ratio and a thinner bilayer would favour the formation of oligomers. White et al. showed that the oligomeric size of solubilised neurotensin 1 receptors depends on receptor concentration [90]. At concentrations below 1 nM, neurotensin 1 receptors would exist as monomers, would bind agonists with a Hill coefficient of ~1 and would readily activate Gq, while at concentrations greater than 20 nM, neurotensin 1 receptors would form dimers, would bind agonists with a Hill coefficient of ~2 and would have a reduced ability to activate Gq. Gavalas et al. showed that, in live cells, untagged protomers of the β2 adrenergic receptor and the µ opioid receptor are not corecruited with affinity-tagged receptors into membrane microdomains, while untagged protomers of the metabotropic glutamate receptor, a class C GPCR, are corecruited with affinity-tagged metabotropic glutamate receptors into the same microdomains [91]. Furthermore, several groups used single-particle tracking to capture the formation and dissociation of class A GPCR dimers on the surface of live cells, and each one
of those studies reports an average lifespan of a GPCR dimer that is in the order of seconds [39,52,53,61].

In view of the studies described above, it seems unlikely that class A GPCRs form stable or constitutive oligomers in live cells. However, the specificity of interactions between different class A GPCRs and the mechanisms that govern those interactions remain poorly understood, and even with a short lifespan, GPCR oligomers still may have a unique pharmacological function and play an important physiological role.
Chapter 4
Future directions
4 Future directions

4.1 Single-particle tracking

4.1.1 Using alternative controls

The results that we obtained from our single-particle tracking experiments are inconclusive, since the single-particle intensity distributions and the trajectories of single-particle intensities over time appear similar for the monomeric control CD86, the dimeric control CD28 and the M₂ muscarinic receptor. One possible explanation for this ambiguity is that our dimeric control CD28, fused with a SNAP-tag at the N terminus, does not form dimers in live cells. While CD28 has been used as a dimeric control in previous SPT [52], FRAP [62] and BRET [76] studies, and CD28 labelled with GFP2 or eYFP at the C terminus show a pattern of FRET efficiencies that is consistent with what is expected for a dimer (Figure 3.10), it is possible that the fusion of a 20 kDA SNAP-tag to the N terminus of CD28 disrupted the proper folding of this receptor and prevented the formation of the intermolecular disulphide bonds. In fact, when we co-transfected cells with CD28 fused with GFP2 or eYFP at the N terminus, we observed low levels of expression and minimal FRET (data not shown), which suggests that the fusion of a fluorophore to the N terminus of CD28 has a detrimental effect on the folding of CD28. In order to measure the oligomeric size of SNAP-tagged CD28 in CHO cells, we could perform western blotting, using an anti-SNAP-tag primary antibody, on solubilised extracts from transiently transfected cells. If we observe that SNAP-tagged CD28 receptors do not form dimers in live cells, we would have to repeat the SPT experiments using other dimeric and oligomeric controls.

One possible alternative to CD28 as a dimeric control would be the signalling threshold-regulating transmembrane adapter 1 (SIT1). SIT1 has been well characterised to form disulphide-linked dimers in live cells [92], and since its extracellular domain is only 15 amino acids long and lacks any known functional motifs [93], it seems likely that it would tolerate well the introduction of a SNAP-tag. Another dimeric control that we could use is the metabotropic glutamate receptor 1, a class C GPCR that also has been shown to exist exclusively as a
disulphide-linked dimer and to tolerate the fusion of a SNAP-tag to its N terminus [38,94,95]. For a tetrameric control, we could use the influenza virus M2 protein, which forms proton channels containing two disulphide-linked dimers [96].

We also could use CD86 fused to two or four SNAP-tags in order to mimic intensities expected for dimeric and tetrameric proteins. Calebiro et al. used CD86 fused to two SNAP-tags as their dimeric control [39], and in their experiments this construct produced single-particle intensity distributions and temporal trajectories that coincided with what was expected for a dimer. In order to be able to manipulate and record the oligomerisation of proteins in a controlled setting, we could fuse the FK506 binding protein (FKBP) to the C terminus of CD86, which would allow us to chemically induce the dimerisation of CD86 with a commercially-available compound AP20187 [97].

4.1.2 Improving the signal-to-noise ratio of the images

Another possible explanation as to why the single-particle intensity distributions and the trajectories of single-particle intensities over time appear similar for our monomeric and dimeric controls is that the quality of our images is not sufficiently high for us to extract individual particles from noise. SNAP-Surface 549 appeared to produce images with a better signal-to-noise ratio than SNAP-Surface 488, but we could not use SNAP-Surface 549 in our single-particle tracking experiments because it showed a high amount of non-specific adsorption to the coverslip surface and to the cell membrane. The cell-permeable dye SNAP-Cell TMR-Star also appeared to produce images of a better quality, but we wished to use a hydrophilic dye that would label only the proteins that are expressed at the plasma membrane. Thus, we resorted to using SNAP-Surface 488 for the body of our work, and the poor signal-to-noise ratio of our images likely lowered the quality of our final results.

SNAP-Surface 488 is a benzylguanine derivative of the fluorescent dye Atto 488. While we could not find other studies that used SNAP-Surface 488 or Atto 488 in SPT experiments, Atto 488 has been used successfully in super-resolution imaging, which also requires bright and
photostable fluorescent dyes [98]. Furthermore, Hern et al. [53] used Alexa Fluor 488 in combination with Cy3B for their dual-colour SPT studies, and Alexa Fluor 488 has comparable photophysical properties to Atto 488 [98]. Thus, performing SPT experiments with SNAP-Surface 488-labelled proteins still should be possible. Nevertheless, fluorophores that emit in the red spectral range, such as Alexa Fluor 647, Cy5 and Dyomics 654, generally are a better suited for SPT. Those fluorophores have similar brightness and photostability to Atto 488 and other green dyes, but the amount of cell autofluorescence is significantly reduced at higher wavelengths [98,99]. This, in turn, leads to better signal-to-noise in the images.

The benzylguanidine derivative of Alexa Fluor 647 is commercially available (SNAP-Surface Alexa Fluor 647, New England Biolabs), and, at present, this appears to be the best choice for labelling SNAP-tagged proteins for SPT experiments. This dye has been used for SPT experiments in the past [39], and the authors do not report having problems with non-specific labelling. However, in order to be able to use this dye, we would have to add an additional laser, capable of emitting in the 600 nm to 700 nm range, to the TIRF microscope that we used for our experiments.

4.1.3 Improving the image analysis pipeline

In this study, we compared three different algorithms, namely GMimPro, uTrack and uTrack with GPUfit, in their ability to detect single particles, and we used uTrack with GPUfit for the body of our work because it appeared to produce the most accurate results, as discussed in Section 2.4.2. However, one prominent single-particle detection technique, the compressed sensing (CS) algorithm [100], was not included in our comparison. CS uses a conceptually different approach than the techniques listed above. Instead of overlaying a Gaussian-shaped mask over individual particles (the approach used by GMimPro), or fitting particles to two-dimensional Gaussians (the approach used by uTrack and GPUfit), CS attempts to find the sparse version of the image that would reproduce most closely the original image after being transformed by the point-spread function of the microscope. This search is formulated as a linear
optimisation problem with quadratic constraints, and it can be specified and solved in Matlab using an external package called CVX [101]. Fitting of individual spots to Gaussians can produce marginally more accurate estimates of single-particle intensities and positions than CS [100], but it requires expression levels of less than 2 molecules/µm², which are low even for stably-transfected cells. CS, on the other hand, can detect most molecules present in the image even at densities as high as 12 molecules/µm² [100]. It is conceivable that if we used CS instead of uTrack with GPUfit, we would be able to detect more particles in our images and the single-particle intensity distributions that we obtain would depend less on the level of expression and in consequence would show less variability from cell to cell.

In order to see if CS is suited better for our work than the other three algorithms, we could use CS to analyse a timelapse movie of a cell expressing our monomeric control CD86 and see if the number of molecules detected by CS correlates more closely with the average intensity in the image. Furthermore, we could simulate images of single particles at different densities and different signal-to-noise ratios, and we could compare the four algorithms in their ability to reproduce the position and the intensity of individual particles. The latter approach would provide us with an estimate of the error that is associated with the detection of single particles, and it also may offer some insight into the origin of the skewness in the single-particle intensity distributions. For instance, if both the real images and the simulated images produce comparably-skewed single-particle intensity distributions, it would suggest that the skewness of the distributions is a consequence of the low signal-to-noise ratio of the images and the algorithms’ inability to separate single particles from noise. If, on the other hand, the single-particle intensities obtained for simulated images are symmetric, it would suggest that the skewness of the distributions obtained for real images is a consequence of the TIRF imaging process, as discussed in Chapter 2.
4.1.4 Dual-colour imaging of receptors and G proteins

There are now four papers that describe the use of SPT to characterise the oligomeric size and the movement dynamics of GPCRs in live cells, and all four papers report that class A GPCRs exist as a mixture of monomers and dimers [39,52,53,61]. It seems unlikely that we would reach a markedly different conclusion if we successfully performed SPT experiments using the M2 muscarinic receptor. However, a possible extension to this work would be to use SPT to characterise the oligomeric size and the movement dynamics of heterotrimeric G proteins and to use dual-colour SPT to observe receptors and G proteins before and after the addition of an agonist.

One approach to performing SPT experiments with G proteins would be to fuse a SNAP-tag or a CLIP-tag [102] to one of the subunits of the heterotrimeric G protein and to label those tags with a cell-permeable dye such as SNAP-Cell TMR-Star or CLIP-Cell TMR-Star. For dual-colour experiments, we could coexpress the M2 muscarinic receptor, or another GPCR, together with the G proteins. Those receptors would be fused to a SNAP-tag at the extracellular N terminus and would be labelled with a cell-impermeable dye such as SNAP-Surface Alexa Fluor 647. If the G proteins are fused to a SNAP-tag, the receptors would have to be labelled before the G protein, so that the cell-permeable dye used to label the G proteins does not label the receptors. If the G proteins are fused to a CLIP-tag, the receptor and the G protein would be labelled simultaneously, since the SNAP-tag and the CLIP-tag are specific to different dyes.

Single-colour SPT experiments would allow us to measure the degree of clustering and the rate and type of diffusion for different G proteins. Dual-colour experiments would allow us to measure the degree of colocalisation between receptors and G proteins, both before and after the addition of an agonist. If there are subpopulations of receptors and G proteins that show distinctly different rates and patterns of diffusion, we could look at how the addition of agonist affects each subpopulation. Hemert et al. [103] used single-molecule epifluorescence microscopy to show that, in Dictyostelium discoideum, approximately 70% of Ga2 and Gβγ exist in a fast-diffusing fraction and the remaining 30% exist in a slow-diffusing fraction. The slow-diffusing fraction was attributed to the Ga2βγ heterotrimer coupled to the cAMP receptor 1 (cAR1). Upon
agonist stimulation, the fast fractions of both Gα2 and Gβγ changed their behaviour from free diffusion to confined diffusion, the slow fraction of Gβγ became highly confined and the slow fraction of Gα2 did not show any change [103]. We could use SPT to see if similar fast and slow fractions are observed for Gαi1 and Gβγ in CHO cells, and to see if the slow fraction can in fact be attributed to a receptor-G protein complex.

4.1.5 Combining FRET with SPT

SPT experiments are limited by the resolution of the microscope, and do not provide information about intermolecular interactions that take place within the diffraction limit. For instance, if a single diffraction-limited spot contains two or more molecules, it does not prove that those molecules are interacting directly with one another, since the molecules may be colocalised within the same microdomain or may be held together by other intermediary proteins. In order to distinguish between colocalisation and true oligomerisation, we could use an approach that combines SPT and FRET on a TIRF microscope. The protein of interest, fused to an acyl carrier protein (ACP) or a SNAP-tag, could be labelled with two distinct fluorophores, and the emission intensity could be split into two channels corresponding to emission from the donor and the acceptor. If colocalisation of two particles labelled with different fluorophores leads to increased emission in the acceptor channel, it would suggest that the two molecules are in sufficient proximity for FRET to occur. If, on the other hand, the excitation of the donor does not lead to emission from the acceptor, it would suggest that the two molecules are colocalised but are not interacting.
4.2 Förster resonance energy transfer

4.2.1 Lowering the level of expression

The results that we obtained using cell-averaged FRET experiments suggest that M2 receptors do not form constitutive oligomers in live cells. However, due to the high level of expression that were produced in transiently-transfected cells, we observe high FRET efficiencies even for our monomeric control CD86, and this makes it difficult to distinguish between monomers and oligomers. This problem may be overcome in part by using stable cell lines. However, the standard approaches for creating stable cell lines, which rely on neomycin (G418) resistance, can be used only to select cells that express either GFP2- or eYFP-tagged proteins, and generating cells that stably express both proteins would be a challenge. An alternative approach would be to create stable cell lines that express the protein of interest, fused to an ACP or a SNAP-tag, and to label those tags with a pair of FRET-compatible synthetic dyes prior to imaging.

We also could try to lower the level of expression that is obtained in transiently-transfected cells. For instance, we could reduce the amount of plasmid DNA used to transfect the cells, we could transfec the cells for a shorter period of time or we could try using different plasmids with a milder promoter. The latter approach appears particularly promising, as the immediate-early cytomegalovirus (CMV) promoter present in the pcDNA3.1 plasmids is one of the most powerful promoters described in the literature [104]. A better promoter for our application may be the human ubiquitin c (UBC) promoter, which has been reported to produce significantly lower levels of expression than the CMV promoter in a variety of cell lines, including a 10-fold lower level of expression in HEK293T cells [104].

If we are successful in achieving lower levels of expression in our cells, the limiting factor may become the low level of signal present in the images. We could improve the signal-to-noise ratio by using the time-resolved FRET (trFRET) approach, where the donors are labelled with a chelated lanthanide dye such as SNAP-Lumi4-Tb and the images are obtained after a delay that is longer than the lifetime of the cell autofluorescence but shorter than the lifetime of the lanthanide dye [105].
4.2.2 Using homo-FRET

The main limitation of measuring FRET between two different fluorophores is that FRET can be observed only for those complexes that have at least one donor and one acceptor. Thus, for a dimeric protein, we can observe FRET for at most one half of all complexes present in the cell, and at an acceptor-to-donor ratio of 1 to 20 we observe FRET for less than 10% of all complexes present in the cell (\(\frac{2 \cdot 1/21 \cdot 20}{21} \approx 0.095\)). Moreover, apparent FRET efficiencies obtained through donor dequenching or acceptor-sensitised emission depend on the acceptor-to-donor ratio, which makes those FRET efficiencies difficult to interpret and compare across different samples.

We could eliminate the dependence of the apparent FRET efficiency on acceptor-to-donor ratio by labelling all proteins with the same fluorophore and using homo-FRET, rather than hetero-FRET, to measure the FRET efficiency. Homo-FRET describes non-radiative energy transfer between two identical fluorophores and is measured typically by illuminating the sample with plane-polarised light and measuring at the anisotropy of the emitted light. Polarised light will selectively excite molecules whose absorption transition dipole moments are aligned with the electric field vector of light, and light emitted by those fluorophores also will have a preferred orientation [106]. Depolarisation of light will occur due to the rotational diffusion of the fluorophores and due to the transfer of energy to nearby fluorophores that have a different orientation. For membrane proteins tagged with organic fluorophores, depolarisation due to rotation occurs typically on a slower time-scale than depolarisation due to FRET, and the loss of anisotropy observed using steady-state measurements is dominated by the loss of anisotropy due to FRET. Furthermore, the two modes of depolarisation can be separated by measuring time-resolved changes in anisotropy [107].

Homo-FRET measurements can be used to look at spatial heterogeneities in FRET efficiencies that are found inside the cell. For example, homo-FRET measurements were used to show that fluorophores bound to the membrane with a glycoprophatidylinositol (GPI) tag form clusters on the plasma membrane but exist largely as monomers in the Golgi apparatus [107]. However, in order to rule out the possibility that the observed FRET efficiencies are an artefact of protein
overexpression and/or clustering, homo-FRET measurements, just like hetero-FRET measurements, must be made at a wide range of expression levels, including low expression levels [97]. Furthermore, homo-FRET measurements include some unique challenges that are absent in hetero-FRET measurements. The intensities obtained in the parallel and perpendicular channels must be corrected for the difference in the sensitivity of the camera to light of different polarities [106]. A certain amount of depolarisation will be caused by the optics of the microscope, and this depolarisation is difficult to correct as it is a function of both the specific optical components and of the position of the pixel in the field of view. A low numerical aperture objective may be used to lower the amount of depolarisation, but this reduces the resolution of the images [108]. Homo-FRET measurements assume that the orientation of the fluorophores is random, and the decrease in anisotropy may be smaller than expected if the orientation of fluorophores in a complex is constrained, as is common for membrane proteins [106]. The intensities measured in parallel and perpendicular channels are subject to Poisson noise and the difference between those intensities often is small. Thus, pixel-level anisotropies that are obtained may have a large standard deviation, which may make them difficult to interpret [106].

Therefore, while homo-FRET measurements would allow us to measure FRET efficiency as a function of expression level only, without having to account for different acceptor-to-donor ratios across different cells, this technique has other limitations which may prevent us from obtaining more accurate results.

### 4.2.3 Estimating acceptor and donor concentrations in real units

One factor limiting the scope of our FRET study is the arbitrary units in which we estimated the total donor and acceptor concentrations ([D] and [A]). This prevents us from obtaining a relevant estimate of the equilibrium association constant (Keq) in the transient dimer model (Equation 26) and the clustering coefficient (k_cor) in the bystander FRET model (Equation 32). Moreover, this prevents us from comparing directly the results of our FRET study with the results that are obtained using other biophysical techniques such as SPT and FCS.
In order to obtain a conversion factor that would allow us to estimate the expression level of donors and acceptors in real units, we could image a solution containing a known concentration of GFP2 or eYFP, using the same settings that are used to image the cells, record the intensity that we obtain for that solution and calculate how many counts per pixel are produced by each molecule per µm². A similar approach was taken by Meyer et al. [75] in order to plot FRET efficiencies as a function of acceptor expression in real rather than arbitrary units.

In theory, FRET measurements can be used to cover a much broader range of expression levels than single-molecule techniques such as SPT and FCS. Thus, it may be possible to use FRET to unite some of the conflicting results that are obtained using other techniques. For example, studies that used SPT to show that class A GPCRs exist as a mixture of monomers and dimers used expression levels of at most several molecules per µm² [52,53] and ideally of less than 0.5 molecules per µm² [39]. On the other hand, a recent study that used FCS to show that several class A GPCRs exist exclusively as dimers used expression levels of 20 to over 200 molecules per µm² [109]. If we observe a low FRET efficiency at expression levels that are used for SPT and a higher FRET efficiency at expression levels that are used for FCS, it would suggest that both the SPT studies and the FCS study are correct and that GPCRs form oligomers in a concentration-dependent manner. If, on the other hand, we observe a similar FRET efficiency at both expression levels, or if the monomeric control CD86 shows a similar FRET efficiency to a class A GPCR while showing distinctly different behaviour in SPT and FCS studies, it would suggest that the results obtained using one of those techniques are being misinterpreted.
References


Appendix A

Appendix A includes three sample images and the fits achieved using the four different models for all donor-acceptor pairs that were investigated in the FRET study. The quality of the fits achieved using the bystander FRET model and the stable dimer model, and the equilibrium association constant predicted by the transient dimer model, were used to make Figure 3.12 and Figure 3.13.
GFP2-M2, eYFP-M2

Figure A.1: FRET efficiencies obtained for CHO cells co-expressing GFP2-M2 and eYFP-M2. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.2: FRET efficiencies obtained for CHO cells co-expressing GFP2-M1 and eYFP-M2. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.3: FRET efficiencies obtained for CHO cells co-expressing GFP2-β2 and eYFP-M2. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
M2-GFP2, CD28-eYFP

Figure A.4: FRET efficiencies obtained for CHO cells co-expressing M2-GFP2 and CD28-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.5: FRET efficiencies obtained for CHO cells co-expressing M2-GFP2 and CD86-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.6: FRET efficiencies obtained for CHO cells co-expressing GFP2-M2<sub>C457A</sub> and eYFP-M2<sub>C457A</sub>. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
**GFP2-M2<sub>C457A</sub>, eYFP-M2**

Figure A.7: FRET efficiencies obtained for CHO cells co-expressing GFP2-M2<sub>C457A</sub> and eYFP-M2. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.8: FRET efficiencies obtained for CHO cells co-expressing Gαi1-GFP2 and M2-eYFP, in the presence of Gβγ. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
**Cav1-GFP2, Cav1-eYFP**

Figure A.9: FRET efficiencies obtained for CHO cells co-expressing Cav1-GFP2 and Cav1-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.10: FRET efficiencies obtained for CHO cells co-expressing GFP2-M2R1 and eYFP-M2R2. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.11: FRET efficiencies obtained for CHO cells expressing MP-GFP2-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.12: FRET efficiencies obtained for CHO cells co-expressing M2-GFP2 and Cav1-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.13: FRET efficiencies obtained for CHO cells co-expressing Gαi1-GFP2 and Cav1-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.14: FRET efficiencies obtained for HEK293 cells co-expressing MP-GFP2 and Cav1-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 79.
Figure A.15: FRET efficiencies obtained for CHO cells co-expressing M2-GFP2 and trFrz1-eYFP. The description of the data presented in each panel and the models that were used to transform and fit the data can be found on page 78.