Insane in the Membrane:
The Functional Assembly of a G Protein Coupled Receptor
at the Single-Molecule Level

by

Yuchong Li

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Department of Physics

University of Toronto

© Copyright by Yuchong Li, 2018
Insane in the Membrane: The Functional Assembly of a G Protein Coupled Receptor at the Single-Molecule Level

Yuchong Li

Doctor of Philosophy

Department of Physics
University of Toronto
2018

Abstract

Many aspects of cellular signaling pathways regulated by G protein coupled receptors (GPCRs) are not completely understood. In particular, two questions have been the focus of much attention and debate: the oligomeric status of the receptor-G protein signaling complex, and the coupling state between the receptor and the G protein in response to external stimuli. Using single molecule fluorescence spectroscopy and microscopy, we investigated these two questions with a focus on the M$_2$ muscarinic acetylcholine receptor and the attendant G$_i$ protein.

The oligomeric status of the M$_2$ receptor and of the G$_i$ protein was first determined using dual-color fluorescence correlation spectroscopy (dcFCS). Positive cross-correlation between differently labeled M$_2$ receptors and differently labeled G$_i$ proteins was observed for samples either purified in detergent solution or expressed in live cells, indicating the existence of oligomeric M$_2$ receptors and oligomeric G$_i$ proteins. The oligomeric sizes of both proteins were then quantitatively measured by single molecule photobleaching (smPB) on a total internal reflection fluorescence (TIRF) microscope. Upon surface immobilization, M$_2$ receptors were found to exist primarily as tetrameric complexes, whereas G$_i$ proteins were found to exist as hexamers in the basal (inactive) state, and
were reduced in size upon binding of GTP. Further evidence of oligomeric M2 receptors was found using smPB in fixed cells, in conjunction with an assessment of the fluorescence brightness analysis of single particles tracked in live cells.

The coupling state between the M2 receptor and the Gi protein was first investigated using dcFCS. In the basal state without the receptor agonist, the amplitude of the cross-correlation was statistically close to zero between co-expressed and differently labeled M2 receptors and Gi proteins. This trend was the same for samples either purified in detergent or expressed in live cells. After receptor agonist was added, the amplitude of the cross-correlation curve increased significantly, indicating that the coupling between the M2 receptor and the Gi protein is transient and only induced by the agonist. The same agonist-induced M2-Gi coupling interaction was also observed in our single particle tracking (SPT) studies of fluorescently labeled M2 receptors and Gi proteins in live cells.
Acknowledgments

The seven years of study towards the PhD degree felt like a marathon to me — there have been multiple ups and downs, both physically and mentally, and even right now near the end I still could not believe that I did it. I sincerely thank all the people who have helped me, enlightened me, encouraged me, and ran alongside me, through this wonderful journey of life. Thank you for being there for me. Words are far from enough to convey my gratefulness.

My special thanks go to my supervisor, Dr. Claudiu Gradinaru. With his deep understanding and broad knowledge in science and in research, he sketched the route map, laid out the road signs, and even helped to clear most of the obstacles for me. Without his guidance, I wouldn’t have been able to make it this far. On top of all the support in the lab, he has become one of my best friends, who was always willing to share in his wisdom and happiness for life. It is awesome to be your student!

Next I would like to thank my current and former fellow lab members Dr. Zhenfu Zhang, Gregory Gomes, Dennis Fernandes, Dr. Baoxu Liu, and Dr. Amir Mazouchi for the patient instructions, fruitful discussions, generous assistance, and relaxing chats. It has been a great pleasure being around you guys in this lab. These days and nights we spent together will be a precious memory that I will cherish for my life.

I would like to express my deep gratitude and appreciations to Dr. James Wells for initiating the collaboration projects, and to all the collaborators in the Wells lab, Dr. Rabindra Shivnaraine, Tina Huiqiao Ji, and Dr. Fei Huang, for their hard work and innovative experimental designs. I would like to thank Dr. Rabindra Shivnaraine again, in particular for his enthusiastic support and motivation in my early days as a clueless new graduate student. I was so lucky to have someone so skilled and experienced to fight through the hardships together.

I would also like to thank my current and former fellow lab members Abdullah Bahram, Jordan Rebelo, Spencer Smyth, Florent Mercier, Charles Huston, Mohamed Salama, John Castroverde, Chris Lee, for their help with my research projects. Thank you all who are
working or have worked in the Gradinaru lab, for creating such a friendly and cooperative atmosphere.

I would like to thank Dr. William Ryu and Dr. Anton Zilman for kindly agreeing to become my supervisory committee members, and for providing invaluable suggestions and advices on my projects throughout the years.

Many thanks to the people from ACGT corp. — Dr. Hai Shiene Chen, Mr. David Coburn, Dr. Omair Noor — for the hard work and the constructive input on the collaboration projects, as well as for the financial support over the last year of my study.

Also thanks to the Department of Physics at University of Toronto, and the CIHR Training Program in Protein Folding and Interaction Dynamics, for the financial support in these years.

And of course, I would like to thank my father, Zhijia Li, and my mother, Haihong Xu, for their endless love. Thank you for giving me life, thank you for bringing me up, thank you for all the lessons about living a valuable and responsible life. Despite being thousands of miles away, you are always my oasis in the desert, and my shelter from the storm.

Last but not least, I would like to thank my friends Ahmad Golaraei, Yao Guo, Yi Han, Richard Kil, Lukas Kotenis, Angel Lai, Xuan Liu, Nafiseh Rafiei, Haowei Wang, Yang Yang, Feng Zhou, and Yige Zhou. The moments we spent together are like the silver lining through the clouds. No matter we are near or distant from each other, I have each of your smiles engraved in my heart.
# Table of Contents

Acknowledgments........................................................................................................ iv
Table of Contents ........................................................................................................ vi
List of Abbreviations .................................................................................................. x

1 Introduction: G Protein Coupled Receptors ................................................................. 1
   1.1 GPCR structure and signaling pathways .............................................................. 1
   1.2 M₂ Receptors and G₃ proteins ............................................................................. 3
   1.3 Project Description and Thesis Outline ............................................................... 4
   1.4 References .......................................................................................................... 6

2 Fluorescence Techniques ............................................................................................ 10
   2.1 Fluorescence Correlation Spectroscopy ............................................................... 11
      2.1.1 FCS theory .................................................................................................. 11
      2.1.2 DcFCS theory ............................................................................................ 15
      2.1.3 The dcFCS microscope in the Gradinaru Lab .............................................. 19
      2.1.4 dcFCS alignment protocol and control measurements .............................. 23
   2.2 Two-Photon Excitation FCS Technique .............................................................. 37
   2.3 SMF Microscopy Techniques .............................................................................. 41
      2.3.1 The TIRF microscope .................................................................................. 42
      2.3.2 Single-molecule photobleaching (smPB) measurements ............................. 43
      2.3.3 Single-particle tracking (SPT) measurements .............................................. 44
   2.4 References .......................................................................................................... 45

3 DcFCS on Receptor-G Protein Complex ...................................................................... 50
3.1 Introduction and Motivation ................................................................................................. 50

3.2 Materials and Methods ....................................................................................................... 51
   3.2.1 Sample preparation ........................................................................................................ 51
   3.2.2 DcFCS Experimental Setup ......................................................................................... 56

3.3 Results and Discussion ....................................................................................................... 59
   3.3.1 Single color FCS experiments in detergent micelles .................................................... 59
   3.3.2 Dual color FCS experiments in detergent micelles ....................................................... 67
   3.3.3 Preparation and control experiments in live cells ......................................................... 75
   3.3.4 Single color FCS measurements in live cells ............................................................... 82
   3.3.5 Dual color FCS measurements in live cells ................................................................. 84

3.4 Conclusions and Future Directions .................................................................................. 97

3.5 References ......................................................................................................................... 98

4 SmPB on Receptor and G Protein Oligomers ...................................................................... 104
   4.1 Introduction ..................................................................................................................... 104

4.2 Materials and Methods ...................................................................................................... 106
   4.2.1 Expression and purification of multiplexed GFP .......................................................... 106
   4.2.2 Preparation of receptor samples and G protein samples ............................................. 107
   4.2.3 Functionality of tagged G proteins and receptors ....................................................... 108
   4.2.4 Total Internal Reflection Fluorescence Microscope ................................................... 109
   4.2.5 Construction and characterization of flow chambers ............................................... 109
   4.2.6 Immobilization of single molecules .......................................................................... 111
   4.2.7 Analysis of TIRF image stacks .................................................................................... 113
   4.2.8 Photobleaching analysis of the intensity-time trajectories .......................................... 114

4.3 Experimental Results ......................................................................................................... 117
   4.3.1 DcFCS Evidence of oligomeric receptor and G protein ............................................. 117
5.3.11 SmPB measurements of M2 oligomeric size in fixed cells..........................167

5.4 Conclusions..................................................................................................169

5.5 Future Directions .........................................................................................170

5.6 References....................................................................................................171

Statement of Contributions ..................................................................................175

List of Publications ..............................................................................................178
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>7TM</td>
<td>seven transmembrane helices</td>
</tr>
<tr>
<td>AIC</td>
<td>Akaike information criterion</td>
</tr>
<tr>
<td>AOTF</td>
<td>acousto-optical tunable filter</td>
</tr>
<tr>
<td>APD</td>
<td>avalanche photo diode</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>Co-IP</td>
<td>co-immunoprecipitation</td>
</tr>
<tr>
<td>cps</td>
<td>counts per second</td>
</tr>
<tr>
<td>dcFCS</td>
<td>dual-color fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>ECL</td>
<td>extra-cellular loop</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>fcd</td>
<td>fraction of co-diffusion</td>
</tr>
<tr>
<td>FCS</td>
<td>fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein coupled receptor kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>ICL</td>
<td>intra-cellular loop</td>
</tr>
<tr>
<td>kcps</td>
<td>kilo counts per second</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>mCh</td>
<td>mCherry (fluorescent protein)</td>
</tr>
<tr>
<td>MP</td>
<td>Myristoylation-Palmitoylation</td>
</tr>
<tr>
<td>MSD</td>
<td>means squared displacement</td>
</tr>
<tr>
<td>NMS</td>
<td>N-methylscopolamine</td>
</tr>
<tr>
<td>OCVF</td>
<td>overlap volume correction factor</td>
</tr>
<tr>
<td>OPE</td>
<td>one-photon excitation</td>
</tr>
<tr>
<td>PoL</td>
<td>precision of localization</td>
</tr>
<tr>
<td>R_H</td>
<td>hydrodynamic radius</td>
</tr>
<tr>
<td>RMS</td>
<td>root mean square</td>
</tr>
<tr>
<td>RMSD</td>
<td>root mean square displacement</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>S.D.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SMF</td>
<td>single molecule fluorescence</td>
</tr>
<tr>
<td>smPB</td>
<td>single molecule photobleaching</td>
</tr>
<tr>
<td>SNR</td>
<td>signal-noise-ratio</td>
</tr>
<tr>
<td>SPT</td>
<td>single particle tracking</td>
</tr>
<tr>
<td>ssDNA</td>
<td>sing stranded DNA</td>
</tr>
<tr>
<td>TIRF</td>
<td>total internal reflection fluorescence</td>
</tr>
<tr>
<td>TPE</td>
<td>two-photon excitation</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultraviolet-visible</td>
</tr>
<tr>
<td>wRSS</td>
<td>weighted residual sum of squares</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
1 Introduction: G Protein Coupled Receptors

G protein coupled receptors (GPCRs) constitute the largest superfamily of proteins encoded by mammalian genomes. The major role of GPCRs is to receive extracellular signals and then initiate downstream intracellular signaling pathways. In human, more than 800 unique GPCRs have been identified, comprising ~4% of the entire protein-coding genome [1]. The physiological processes that involve GPCRs include the visual sense, the gustatory sense, the olfactory sense, behavioral and mood regulation, immune system regulation, and nervous system regulation, etc. [2, 3]. It has also been reported that GPCRs are mediating the growth and metastasis of some types of cancer cells [4, 5]. This versatility in function allows GPCRs to be the targets of more than 30% of all modern drugs, with the potential to treat dozens of diseases and health conditions, among which some notable examples are Diabetes, Parkinson’s disease, cardiovascular disease, schizophrenia, depression, drug addiction, and obesity [6-8].

1.1 GPCR structure and signaling pathways

The most prominent structural feature shared by all GPCRs is the seven transmembrane helices (7TM), with an extracellular N-terminus and an intracellular C-terminus [9]. The seven transmembrane helices are connected by three extracellular loops (ECLs) and three intracellular loops (ICLs). The ECLs are highly involved in the ligand binding process [10]. The ECL1 and ECL3 are short and well conserved for different receptors, while the ECL2 displays a more diverse structure and is believed to be responsible for the specificity of ligands [11-13]. The ICLs interact with intracellular signaling proteins such as G protein and arrestin [14, 15]. Generally speaking, the ICLs are more conserved compared to the ECLs, possibly due to less variety in the binding targets [16]. The ICLs are also more flexible, displaying significant conformational changes upon receptor activation and G protein coupling [17].

The G proteins that interact with GPCRs are a class of proteins that are all made up of three subunits: α, β, and γ. The three subunits can either be coupled as a complex (also
known as the G protein heterotrimers), or be separated into two units (namely the Gα subunit and the Gβγ heterodimer) [18].

GPCRs exist at the interface of a cell’s external and internal environments. When the matching natural ligand—amine, ion, nucleoside, lipid, peptide, protein, or photon—binds to the receptor’s active binding site, the receptor undergoes a conformational change to form its active state[3]. The activated receptors are able to interact with G proteins (for signaling transduction) or arrestins (for desensitization) inside the cell. When the receptor interacts with a G protein, it promotes the α subunit of the G protein to release the GDP (guanosine diphosphate) that was bound to the α subunit for a GTP (guanosine triphosphate) [19]. With the binding of GTP, the Gα subunit dissociates from the β and γ subunits [20, 21]. Both the Gα-GTP and Gβγ heterodimer are then able to act on downstream effectors, thus setting start to a specific cellular mechanism [14, 22]. When the receptor is exposed to persistent stimulus, the C-terminus of receptor can be phosphorylated by G protein coupled receptor kinases (GRKs) and then bound to arrestin. The receptors that are coupled with arrestin will be blocked from further interaction with G proteins, and targeted for internalization (i.e. receptor recycling pathways).

G proteins are water soluble proteins. With the help of post-translational modifications of myristolation and/or palmitoylation, G proteins are typically anchored to the cell membrane [23, 24]. The G proteins act as molecular switches that are either in the “basal state” when bound to GDP, or in the “activated state” when bound to GTP. In the basal state, the GDP-Gα subunit forms a stable heterotrimer with Gβγ subunits. This Gα/Gβγ interaction serves to enhance localization to the membrane, and to slow the spontaneous dissociation of GDP [25, 26]. Upon the activation by a GPCR and the exchange of GDP for GTP, the Gα undergoes dramatic conformational changes in its switch regions, resulting in the dissociation from the Gβγ, and a decreased affinity to the GPCR [21, 26]. As a weak GTPase, the GTP-Gα subunit hydrolyzes the GTP into GDP at a rate of 0.1–5 min⁻¹ [27]. The GDP-Gα eventually decouples from the effector, re-couples with the Gβγ, thus completing a G protein signaling cycle [28].
1.2 M₂ Receptors and Gᵢ proteins

The subtypes of GPCR and G protein that are studied in the current research projects are the M₂ muscarinic acetylcholine receptor and the Gαᵢβ₁γ₂ protein, respectively.

The M₂ muscarinic acetylcholine receptor, or the M₂ receptor, is a member of the largest GPCR family—the Class A (rhodopsin-like) GPCRs. In humans, it is encoded by the CHRM2 gene, and expressed in heart cells [29]. After binding to acetylcholine, which is released from the parasympathetic nerves, the M₂ receptors act to slow down the heart rate to normal sinus rhythm (between 50 to 100 beats per minute), reduce contractile force of the atrial cardiac muscle, and reduce the conduction velocity of the atrioventricular node [30, 31].

The functionality of the M₂ receptor functions is contingent upon binding to the Gᵢ proteins. The Gᵢ-α subunit, when bound to a GTP, decreases the activity of various Cyclic adenosine monophosphate (cAMP) dependent pathways [32, 33]. The Gβγ subunit, on the other hand, can open the K⁺ channels in the parasympathetic notches of the heart, causing an outward current of K⁺, which slows down the heart rate [34] [35].

The M₂ receptor is one of the five subtypes of muscarinic acetylcholine receptors [36]. Despite decades of effort, most drugs developed thus far for muscarinic receptors are not clearly selective for subtypes, possibly due to their high degree of sequence homology [37, 38]. However, the M₂ receptor is distinctive from the others for its specific allosteric activity, that is, the ability to bind to certain ligands specifically at a secondary (allosteric) site and thus modulate the affinity of the ligand binding to the primary (orthosteric) site of the receptor. For this reason, the M₂ receptor is of particular interest for drug development, as well as for studying the specificity of ligand-receptor interactions and the mechanism of allosteric modulation in GPCRs [30].

One of the key aspects of the M₂ receptor signaling mechanism is the coupling interaction between the receptor and the Gᵢ protein in response to external stimuli. This interaction dictates the response time and signal efficiency of the receptor signaling process. A pre-coupled receptor-G protein complex would allow for rapid and specific response but limit
the total number of G proteins that a receptor may have access to; whereas a system containing independently diffusing and transiently interacting receptors and G proteins has the potential for high signal amplification but could be limited in response time. Differentiating between a pre-coupled complex and a transiently interacting crowd would provide valuable insights into the activation of the receptors.

Another important aspect of the M2 receptor signaling process is the oligomerization status of the receptors and of the Gi proteins. The oligomerization of receptors has been a topic of much debate. Oligomeric receptors could allow for inter-molecular modulation of the functioning of individual molecules, and open up possibilities of subtype-specific therapeutic designs. Oligomeric G proteins, on the other hand, are likely related to their signaling efficiency, but have been out of the scope of most pharmacological and pharmaceutical studies.

This thesis sheds light on the abovementioned two aspects of the M2 receptor-Gi protein signaling complex. A multitude of techniques, in particular the fluorescence spectroscopy and microscopy techniques that are available in the Gradinaru Lab, were employed to attack these question from various directions.

1.3 Project Description and Thesis Outline

All projects described in this thesis were the result of a collaboration with the Wells Lab in the Department of Pharmaceutical Sciences, University of Toronto. The two major questions being investigated are: (1) the size of oligomerization of M2 receptors and of Gi proteins, and (2) the interaction between the M2 receptor and the Gi protein before/after receptor activation.

For these studies, fluorescent protein-fused M2 receptor and Gi protein sequences were modified and inserted into their respective DNA plasmids by the Wells Lab. The detergent-solubilized M2 receptors and Gi proteins samples were extracted and purified in the Wells Lab. The live-cell M2 receptors and Gi proteins samples were expressed in live cells in the Gradinaru Lab. Quality-control and functional biochemistry assays (Western
Blot, radioligand binding, etc.) were carried out in the Wells Lab. The experiment design, fluorescence measurements, and data analysis were performed in the Gradinaru Lab.

Chapter 2 lays out the techniques and equipment that were used for the data presented in this thesis, with a focus on the dual-color fluorescence correlation spectroscopy (dcFCS) setup that was designed and built by me. The theoretical background and the optical alignment protocol of the dcFCS technique are described in this chapter. In particular, the dcFCS calibration procedures that were developed to correct for various artifacts are provided in a step-by-step manner. In addition, key information regarding the total internal reflection fluorescence (TIRF) microscope that was used for single-molecule photobleaching (smPB) and single particle tracking (SPT) assays is briefly described.

Chapter 3 presents the results of dcFCS studies of \( M_2 \) receptors and \( G_i \) proteins, both purified in detergent solution, and expressed in live cells. Details of the sample preparation protocols are provided. With a series of control measurements, oligomers of \( M_2 \) receptors and oligomers of \( G_i \) proteins were qualitatively and unambiguously identified. Furthermore, the \( M_2 \) receptor and \( G_i \) protein were found to be separated in their basal state and only coupled after the receptor was activated by agonist, favoring the \( G \) protein recruitment interaction scenario.

Chapter 4 presents the theory and results of the single-molecule photobleaching stoichiometry (smPB) studies of the size of oligomerization of \( M_2 \) receptors and \( G_i \) proteins. The single-molecule immobilization method was implemented in the Gradinaru Lab with low background fluorescence and high specificity. The change-point analysis that was previously developed by Dr. Zhenfu Zhang from the Gradinaru Lab was applied to identify photobleaching steps of the intensity traces of surface-immobilized molecules. Fused green fluorescence protein (GFP) multiplexes were also examined to characterize the photobleaching events. Based on the distribution of photobleaching steps and step intensities, the GFP-tagged \( M_2 \) receptors were estimated to be tetramers, while \( G_i \) proteins were estimated to be hexamers.

Chapter 5 presents the methods and results of the single particle tracking (SPT) studies of \( M_2 \) receptors and \( G_i \) proteins in live cells. The TrackMate software was adopted for
tracking the trajectories for the diffusion of GFP- or mCherry-tagged M₂ receptors and Gᵢ proteins. Mean squared displacement (MSD) of the tracked trajectories were computed and first fitted to a general anomalous diffusion model. The anomalous factor categorized the diffusion behaviors of the particles into sub-diffusion, normal diffusion, and super-diffusion. The sub-diffusion regime was compatible with the hypothesis of confinement domains formed by a membrane actin meshwork. The super-diffusion regime was compatible to active transportation by myosin-actin motors. The diffusion properties of the Gᵢ proteins in all three regimes were found to be highly distinctive from those of the M₂ receptors when in the absence of agonist (i.e. inactive state); however, when the receptor agonist was introduced, the diffusion properties of the Gᵢ proteins became similar to those of the M₂ receptors. This observation strongly favors the receptor-G protein recruitment interaction model. Lastly, the fluorescent complexes most likely consisted of more than one single monomeric unit, as inferred from an intensity analysis of the tracked particles in live cells and of the immobilized particles in fixed cells.

1.4 References


2 Fluorescence Techniques

Membrane proteins have always been a great challenge for researchers. It is estimated that more than 30% of the human genes are encoding for membrane proteins [1]. Currently among more than 120,000 resolved protein structure entries in the RCSB Protein Data Bank, less than 3,000 are of membrane proteins, representing slightly more than 600 unique proteins. Studies on membrane proteins began later and have progressed slower than those on water soluble proteins [2-4]. The main difficulties include, but not limited to, lower production yields, more stringent requirements for the purification conditions and higher tendency for denaturation [5]. These difficulties impose serious constraints on the biophysical techniques that can be used to study membrane proteins, since they have to be sensitive (i.e., operating at low, sub-micromolar concentrations), robust, reliable, and relatively fast, delivering results in less than few hours to minimize the influence of denaturation.

Fluorescence techniques possess a series of advantages that meet the above requirements [6]. In the process of fluorescence, a fluorophore (e.g. a fluorescent dye or protein) absorbs photons of light of a certain wavelength and then emits photons with a longer wavelength. This change in the wavelength between the excitation and the emission spectra is known as the Stokes shift [7]. The Stokes shift of the fluorescence signal relative to the excitation light allows for insertions of spectral filters, i.e., long-pass and/or band-pass optical filters, which rejects the scattered light and the signal from impurities, thus minimizing the background and yielding a high signal-to-noise ratio. This ratio can be further improved through site-specific labeling of the target protein with bright photostable organic dyes. Combined with low dark noise, high efficiency single-photon counting detectors, the sensitivity of fluorescence techniques can ultimately reach the single-molecule level [8, 9].

Throughout this thesis, two single-molecule fluorescence (SMF) spectroscopy techniques are employed for dissecting the physical properties of transmembrane G protein coupled receptors (GPCRs) and attendant G proteins: dual-color fluorescence correlation
spectroscopy (dcFCS), and Total Internal Reflection Fluorescence (TIRF) Microscopy. The two SMF techniques are explained in detail in this chapter.

2.1 Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) is a well-developed non-invasive technique for measurements of nanosecond-to-millisecond time-scale fluctuations in the intensity of the fluorescence signal. It can provide information on the diffusion hydrodynamic size of fluorescently labelled biomolecules, internal conformational dynamics, binding kinetics and changes in the local biological environment [10]. Due to ease of use and versatility, FCS and its variants have been popular techniques for the studies of GPCRs and other membrane proteins both in purified form and in live cells [11-14]. In the beginning of this Chapter, the theory of correlation spectroscopy is briefly introduced, followed by a detailed description of the dual-color fluorescence correlation spectroscopy (dcFCS) theory, which is a versatile extension of the FCS technique. Next is a comprehensive description of the dcFCS microscope that I constructed in the Gradinaru lab, followed by the calibration principles and the operation procedures for our customized setup.

2.1.1 FCS theory

FCS is a biophysical method which quantifies the fluctuations of the emission intensity of fluorescent molecules diffusing randomly through an optically confined illumination field [15]. The fluctuations are analyzed with the method of correlation, by which the temporal similarity of two time-dependent signals is evaluated, yielding a correlation function. Typical factors that affect the correlation function include the local concentration, the diffusion of the labeled molecules, conformational fluctuations, binding processes, molecular crowding, spatial confinement and dimensionality, and last but not least the photophysics of the fluorophore [13, 16].

FCS experiments are usually performed on a confocal fluorescence microscope, with an emission pinhole in front of the photon detector to create a diffraction-limited detection volume at the objective in the sample. The detection volume typically takes on the shape of a 3D Gaussian ellipsoid, which can be described by [15, 17]:

11
\[ \Omega(x, y, z) = \frac{2^{3/2}}{\pi^{3/2} w_0^2 z_0} \exp \left\{ -2 \left( \frac{x^2 + y^2}{w_0^2} - 2 \frac{z^2}{z_0^2} \right) \right\} \]  

(Equation 2.1)

where the \( w_0 \) and \( z_0 \) are the lateral and axial \( 1/e^2 \) radii of the Gaussian profile. The effective volume of the 3D Gaussian profile is thus given by \( V_{\text{eff}} = \pi^{3/2} w_0^2 z_0 \). Thanks to the high-numerical-aperture objective and the lasers with visible wavelength (400–700 nm), the detection volume can be as small as 1 femtoliter. Such a well-defined detection volume provides high signal-to-background ratio for (quasi) single-molecule fluorescence detection, and high 3D spatial resolution for confocal fluorescence imaging. The fluorescence signal originated far from the center of the detection volume is effectively rejected, improving spatial resolution and the signal-to-noise ratio. In addition, single-photon counting detectors, such as avalanche photo-diode (APD) detectors are employed together with either pulse-based counting electronics or with hardware correlators to push the time resolution to the low nanosecond region. This ensures a time window of at least 9 orders of magnitude, which can capture a wide range of photophysical and physiological processes.

An autocorrelation function is built either in the software or in the hardware based on the fluctuations of the measured fluorescence signal. The mathematical equivalent of the process of autocorrelation construction is in the form of

\[ G(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2} \]  

(Equation 2.2)

where the \( \delta I(t) \) indicates the fluctuation in the fluorescence intensity \( I \) at time \( t \) with relative to its mean value \( \delta I(t) = I(t) - \langle I(t) \rangle \); \( \delta I \) is multiplied with itself shifted along the time axis by a “lag time” \( \tau \), then averaged by integrating over the whole time span of the experiment, and finally normalized by dividing the square of the mean intensity value. Therefore the autocorrelation is essentially quantifying the self-similarity of a signal with time. In practice, in our lab, the fluorescence signal is acquired as discrete single photon events by the APD detectors, thus enabling a more computationally efficient approach to build the correlation function in real time [18].
Multiple autocorrelation functions are repeatedly collected on the same sample for calculating the uncertainty (standard deviation function) in the correlation function at each lag time point. Weighted nonlinear regression is then performed on the correlation function (with the standard deviation function serving as the weights) to extract physical parameters of the sample and the fluorophore.

Figure 2-1. Illustration of the data acquisition process of a single-color FCS auto-correlation function. (A) Fluorescent molecules diffuse through the 3D Gaussian detection volume created by a confocal microscope. (B) The fluorescence intensity trace is collected by the detector, and correlated with itself after shifting along the time axis by a lag time \( \tau \). (C) The resultant FCS function is then fitted to an appropriate analytical model to retrieve molecular parameters of interest (according to Equation 2.3).

Theoretical FCS regression models can be derived based on the assumptions of: a 3D Gaussian excitation profile, Poisson distribution of the fluorescence process, and that fluctuations in the local concentration of the fluorophores are governed by 3D diffusion laws [15]. The major component of an FCS regression model is the diffusion term. The diffusion term allows for the estimation of important sample parameters such as the local concentration, the diffusion coefficient and the diffusion mode. Depending on the nature of the sample, the diffusion term can take on different forms as either 2D or 3D diffusion, single- or multi-component diffusion, normal or anomalous diffusion. In addition to the diffusion term, one or more photo-blinking terms may be needed to account for the
transient dark states of the fluorophores, e.g., transition of the exited electron to triplet states or quenching by proximal aromatic groups. Taking the 3D 1-component-normal-diffusion-1-triplet model as an example, the second order autocorrelation function $G(\tau)$ can be analyzed according to the following theoretical form:

$$G(\tau) = \frac{1}{\langle N \rangle} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \left( 1 + \frac{1}{s^2} \cdot \frac{\tau}{\tau_D} \right)^{-\frac{1}{2}} \left( 1 + \frac{f_{\text{trip}}}{1 - f_{\text{trip}}} \exp \left\{ -\frac{\tau}{\tau_{\text{trip}}} \right\} \right)$$  

(Equation 2.3)

where $\langle N \rangle$ denotes the average number of molecules in the detection volume; $\tau_D$ is the average transit time of the molecule through the detection volume; $s$ is the aspect ratio describing the 3D Gaussian detection volume $s = z_0 / w_0$; $\tau_{\text{trip}}$ is the triplet lifetime and $f_{\text{T}}$ is the population fraction of the triplet state of the fluorophore. The triplet term describes the common photophysical process in which the excited fluorophores temporarily enters a dark state by transitioning into non-radiant triplet states [15, 17].

To estimate equipment-independent parameters such as the concentration and the diffusion coefficient of the molecule, the dimensions of the confocal detection volume (namely $w_0$ and $z_0$ as in Equation 2.1) have to be determined by measuring the autocorrelation functions of calibration dyes with known diffusion coefficients (such as Rhodamine 6G, Rhodaminene 110, etc.) [19-21]. The fitting model for retrieving the dimensions of the confocal detection volume with calibration dyes is in the form of Equation 2.4 below:

$$G(\tau) = \frac{1}{\langle N \rangle} \left( 1 + \frac{4D\tau}{w_0^2} \right)^{-1} \left( 1 + \frac{1}{s^2} \cdot \frac{4D\tau}{w_0^2} \right)^{-\frac{1}{2}} \left( 1 + \frac{f_{\text{trip}}}{1 - f_{\text{trip}}} \exp \left\{ -\frac{\tau}{\tau_{\text{trip}}} \right\} \right)$$  

(Equation 2.4)

where $D$ denotes the diffusion coefficient of the calibration dye. The relation $w_0^2 = 4D\tau_D$ for free 2-dimensional diffusion has been applied to expand $\tau_D$ in Equation 2.3.

There are multiple advantages that the FCS technique can offer for the study of membrane receptor proteins. First of all, it minimizes sample consumption. In fact, ultralow sample concentrations of 1~10nM are the most desirable conditions for FCS
measurements because the fluorescence fluctuations from single fluorophores will not be averaged out by neighbor molecules within the detection volume. If longer data acquisition times are feasible, e.g. on the time-scale of hours, then the FCS technique is applicable across a large range of concentrations, 0.01 nM – 1 µM [22]. The higher limit is comparable to the levels of proteins expressed in live cells. Another advantage of FCS over other biophysical techniques for protein studies is the ease of performing measurements. An FCS measurement does not impose many limitations on the sample preparation, nor does it require constant attention from the operator during the data acquisition. Difficulties are mostly encountered before the experiment, in the setup alignment stage, and after the experiment, with respect to understanding which models should be chosen for data analysis [23].

The setup alignment phase is aimed at achieving, as best as possible, a diffraction limited, 3D-Gaussian detection volume for freely diffusing fluorescent molecules. Nearly all the analytical FCS fitting models used for practical applications assume a 3D-Gaussian distributed detection volume with axial symmetry. Deviations from the Gaussian profile, possibly due to imperfect excitation and/or detection light beams, will give rise to unexpected fluorescence fluctuations, and thus require more complicated fitting models for accurate interpretation of the data [23, 24].

The analysis of the experimental correlation functions also demands careful considerations. One needs to be aware of the potential underlying physical processes that could give rise to fluorescence intensity fluctuations for each specific sample. The choice between various fitting models should reflect the underlying processes in order to prevent mis- and over-interpretations of the data. Inferior fitting quality (such as high reduced $\chi^2$ value and correlated residuals) or non-physical fitting parameters are often indicators of an inappropriate fitting model.

### 2.1.2 DcFCS theory

In the case of dual- or multi-color FCS, the setup contains more than one excitation wavelength and more than one spectral detection channels are involved. For clarity, throughout the following discussion of the dual-color FCS theory, excitation laser lines
will be named using their intrinsic colors and the wavelength, (e.g. the “green 532 nm laser”). Fluorophores will be named by the color of the peak emission spectrum (e.g. the Green Fluorescent Protein, GFP, is a green fluorophore). The two detection channels will always be named as “green detection channel” and “red detection channel”, regardless of the exact colors of the fluorescence signals that are reaching the detectors. The reason is that there will always be two and only two detection channels that are set to selectively collect the emission signals from two different fluorophores.

Figure 2-2. Illustration of the acquisition process of a complete set of dcFCS functions. (A) Double-labeled molecules diffuse through the detection volumes created by two independent lasers and detection pinholes. (B) Intensity traces collected by the green and red detection channels. (C) Two auto-correlation and one cross-correlation functions can be constructed from the two intensity traces. The resultant FCS functions can then be fitted globally to retrieve local concentrations of the green and the red species, and the fractions of co-diffusing species (Equation 2.7).

A dual-color fluorescence correlation spectroscopy (dcFCS) microscope was constructed in the Gradinaru lab as a major extension to the single-color FCS. The dcFCS technique employs two spectrally separated fluorescent labels, allowing for simultaneous measurements of two diffusing species. The cross-correlation between the two fluorescent signals contains direct and unambiguous information about the interaction between the two species. Together with the single-color autocorrelation functions which are collected at the same time, dual-color cross-correlation data is essential for extracting
equilibrium state properties in inter-molecular binding and dynamic co-localization processes [25, 26].

The outputs for dcFCS experiments are three correlation functions [27, 28]: two autocorrelations corresponding to each spectral species and one cross-correlation between them. The two auto-correlations \(G_g, G_r\) can be calculated directly from the time-dependent fluorescence intensity fluctuations detected in the green \((\delta I_g)\) and the red \((\delta I_r)\) channels upon green and red excitation, respectively:

\[
G_i(\tau) = \frac{\langle \delta I_i(t) \cdot \delta I_i(t+\tau) \rangle}{\langle I_i(t) \rangle^2}, \quad i = g \text{ or } r \quad \text{(Equation 2.5)}
\]

The experimental cross-correlation function can be calculated based on the same two signals according to:

\[
G_x(\tau) = \frac{\langle \delta I_g(t) \cdot \delta I_r(t+\tau) \rangle}{\langle I_g(t) \rangle \langle I_r(t) \rangle} \quad \text{(Equation 2.6)}
\]

Taking the 3D normal 1-component-diffusion-1-triplet model as an example, the experimental auto-correlation and cross-correlation functions can be analyzed by globally fitting with the theoretical model [27]:

\[
G_g(\tau) = G_g(0) \left(1 + \frac{\tau}{\tau_{D,g}}\right)^{-1} \left(1 + \frac{\tau}{\tau_{D,g}} \cdot \frac{\tau}{\tau_{D,g}}\right)^{-\frac{1}{2}} \times Triplet_g
\]

\[
G_s(\tau) = G_s(0) \left(1 + \frac{\tau}{\tau_{D,r}}\right)^{-1} \left(1 + \frac{\tau}{\tau_{D,r}} \cdot \frac{\tau}{\tau_{D,r}}\right)^{-\frac{1}{2}} \times Triplet_r
\]

\[
G_s(\tau) = G_s(0) \left(1 + \frac{\tau}{\tau_{D,s}}\right)^{-1} \left(1 + \frac{\tau}{\tau_{D,s}} \cdot \frac{\tau}{\tau_{D,s}}\right)^{-\frac{1}{2}}
\]

with \(Triplet_i(\tau) = 1 + \frac{f_{trip,i}}{1 - f_{trip,i}} \exp\left\{\frac{-\tau}{\tau_{trip,i}}\right\}, \quad i = g \text{ or } r \)
where the initial correlation amplitudes $G_g(0)$, $G_r(0)$, and $G_x(0)$ were used instead of the $< \!N_g \!>$, $< \!N_r \!>$, and $< \!N_x \!>$ values (average number of molecules in the detection volume) for the convenience of subsequent calculations (e.g. Sections 0 and 2.1.4.7). The relation between the initial cross-correlation amplitude $G_x(0)$ and the average number of co-diffusing dual color molecules in the detection volume $< \!N_x \!>$ is as follows:

$$G_x(0) = \frac{< \!N_x \!>}{< \!N_g \!>< \!N_r \!>} \quad \text{(Equation 2.8)}$$

Equation 2.8 indicates that the amplitude of the cross-correlation function is directly proportional to the number of co-diffusing (or dual-color) molecules, $< \!N_x \!>$, and inversely proportional to the number of each single-color species, $< \!N_g \!>$ and $< \!N_r \!>$. If we define $c_g$, $c_r$ as the molar concentrations of species that contain green and red labels, respectively, the number of molecules in the detection volume for single- and dual-color species can be written as:

$$< \!N_i \!> = c_i \cdot V_{eff,i} \cdot N_A, \quad \text{where } i = g, r, \text{ or } x \quad \text{(Equation 2.9)}$$

Considering that the auto-correlation amplitudes $G_g(0) = \frac{1}{< \!N_g \!>}$ and $G_r(0) = \frac{1}{< \!N_r \!>}$, the fraction of co-diffusion ($fcd$) for each fluorophore can be expressed as:

$$fcd_g \equiv \frac{c_x}{c_g} = \frac{G_x(0)}{G_g(0)} \cdot \text{OVCF}_g,$$

$$fcd_r \equiv \frac{c_x}{c_r} = \frac{G_x(0)}{G_g(0)} \cdot \text{OVCF}_r$$

where $\text{OVCF}_g \equiv \left( \frac{V_{eff,g}}{V_{eff,x}} \right)$; $\text{OVCF}_r \equiv \left( \frac{V_{eff,r}}{V_{eff,x}} \right)$.  

The two-color “overlap volume correction factors” $\text{OVCF}_g$ and $\text{OVCF}_r$ must be determined independently, but under the same experimental conditions, in order to be able to estimate the fractions of co-diffusing species. Details about the calibration
measurements for determining the overlap volume are discussed in section 0 and they involve dcFCS measurements on standard dual-labelled DNA samples species.

2.1.3 The dcFCS microscope in the Gradinaru Lab

I designed and built the dcFCS microscope setup in the Gradinaru Lab (Figure 2-3) to facilitate studies on intra- and inter-molecular interactions and to provide quantitative assessment of dye labeling of proteins for single-molecule fluorescence experiments. The setup achieves extended versatility and superior sensitivity by utilizing three laser lines: Blue (TECBL-488nm, WorldStarTech, Canada), Green (MatchBox2-532nm, IntegratedOptics, Lithuania) and Red (TECRL-633nm, WorldStarTech, Canada); and four single photon avalanche diode detectors (SPCM-AQR-13-FC, PerkinElmer Inc., USA; “APD-1” ~ “APD-4” in Figure 2-3). A triple-band laser excitation dichroic (FF494/540/650-Di01, Semrock, USA; “Di-ex” in Figure 2-3) and a high-quality emission dichroic (FF585-Di01, Semrock; “Di-em” in Figure 2-3) were selected for simultaneous multi-color excitation and detection. Through the incorporation of a high-numerical-aperture objective (1.4/100X UplanSApo, Ol ympus, Japan), a 100x100x50 μm (x-y-z) 3D precision piezo stage (Nano-LP100, Mad City Labs) and a 4-channel hardware correlator (Flex11-8CH, correlator.com, China), the microscope is capable of nm-precision scanning confocal fluorescence imaging and real-time multi-channel FCS measurements with nanosecond resolution.
Figure 2-3. Schematic optical layout of the multi-color dcFCS microscope in the Gradinaru Lab. Color codes “b”, “g”, and “r” denote blue, green, and red, respectively.

Flex11-8Ch is an advanced multiple-tau digital correlator that was custom-built for our lab by correlator.com with capabilities for multi-channel detection, auto- and cross-correlation, direct intensity trace output, detector overload protection, and confocal scanning. The temporal resolution of the correlation functions is 1.5 ns. It features eight BNC ports, of which ports 1–4 are the input channels receiving signals from the four single photon counting APD detectors (Figure 2-3). Ports 5–7 are TTL gating outputs that shut off an APD detector if its photon count rate measured in the corresponding Flex channel exceeds 1MHz. Port 8 is the scanning pixel clock input receiving synchronization clock pulses from the piezo stage driver. During an image scan, the continuous photon count signal acquired in Ports 1–4 are packaged into pixelated counts according to these pixel clock pulses received from the piezo driver. The Flex correlator is connected to the computer via a USB port and communication can be established either using the manufacturer’s software or a LabVIEW Dynamic Link Library (flex11_8ch.dll).
Figure 2-4. A screenshot of the LabVIEW controller program “dcFCS online” during an FCS experiment. On the left side, the panel shows the real-time fluorescence count-rates, the correlation functions, the count-rate trajectory history, and on the bottom right, the scan image of the fluorescent bead for the latest alignment.

I developed a graphical user interface for data acquisition on the dcFCS setup based on the LabVIEW system design software (National Instruments, USA) and the dll library provided by correlator.com (Figure 2-4). The program integrates all the necessary functions to perform scanning fluorescence imaging and fixed-position multi-channel FCS measurements, including changing the correlator operational mode, moving the piezo stage, and monitoring the real time count rate on the single-photon detectors.

I also developed a graphical user interface (Figure 2-5) for conveniently viewing previously acquired data and combining separate files from the same sample into one processed file that is ready for regression analysis. The program allows the user to visually screen through all the data and discard any undesirable measurements. Upon completion of the screening, the program calculates the averaged correlation function,
\( \bar{G}(\tau) \); and then the standard deviation at each lag time point from the averaged correlation function, \( S.D.(\tau) \), according to:

\[
S.D.(\tau) = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (G_i(\tau) - \bar{G}(\tau))^2}
\]

(Equation 2.11)

where \( N \) is the total number of data files, and the subscript \( i \) is the “file index” assigned to each data file after importing.

**Figure 2-5.** Screenshot of the custom LabVIEW program developed in our lab to display FCS functions and to combine multiple FCS curves measured on the same sample. After selecting multiple data files collected from the same sample, the top panel shows the FCS functions stored in the current raw data file specified by the “file index”. The bottom panel provides a preview of the averaged FCS curve.
The program is also capable of normalizing the FCS curves so that the amplitudes at 1μs lag time $G(1\mu s)$ are scaled to unity (1μs has been arbitrarily chosen as a lag time 10 times shorter than typical time-scales of most molecular level diffusion motion). Normalizing the FCS curves effectively eliminates the influence of sample concentration, and thus facilitates comparison of the diffusion properties between different samples.

2.1.4 dcFCS alignment protocol and control measurements

2.1.4.1 Principles of one-color alignment

An optimal two-color alignment builds upon an optimal one-color alignment. Starting from the laser output and along the optical path, a good one-color alignment should exhibit the following properties:

(1) Optimal 3D position of the excitation pinhole along the excitation beam path to maximize transmission efficiency;

(2) The excitation beam passes through the center of all reference irises;

(3) The excitation beam is incident at 45° on the excitation dichroic; the reflected beam is collinear with the optical axis of the objective and perpendicular to the plane of the sample;

(4) The emission dichroic, long-pass, and band-pass filters are selected so that background rejection is maximized and the signal loss is minimized;

(5) The size of the pinhole in the emission path should be around one Airy unit

$$d_{\text{Airy}} = \frac{1.22\lambda}{\text{NA}} \cdot M \cdot 3.6$$  \hspace{1cm} \text{(Equation 2.12)}

where $\lambda$ is the wavelength of the laser, $M$ is the magnification of the objective, NA is the numerical aperture of the objective.

(6) Optimal 3D position of the emission pinhole and of the APD detectors along the fluorescence emission path to maximize the signal detected.
The requirements (1)–(3) above are completed with the help of irises and pre-determined reference points around the setup. The requirement (6) above can be completed with a coverslip coated with sparse fluorescent beads (TetraSpeck Microspheres, 0.1 μm, ThermoFisher T7279). The coverslip of the beads is first placed on the sample stage, and then the positions of the APD detectors and of the emission pinhole are adjusted so that the fluorescence signal on the detectors are maximized. The complete alignment procedure has been described in detail in the lab protocol.

2.1.4.2 Principles of two-color alignment, optimizing overlap volume

For dcFCS, two fluorophores with different spectral properties are excited simultaneously using two different laser lines. Based on Equation 2.6, the measured co-diffusion fraction depends on the overlap between the two detection volumes. Therefore, an optimized two-color volume overlap is essential for efficient cross-correlation measurements [28].

Two-color volume overlap is challenging to achieve because chromatic aberrations of the optical elements along the light path could add up to a significant difference in the dimensions and positions of the focal volumes of two lasers with different wavelengths. Achromatic lenses and an apochromatic objective have been employed in the dcFCS microscope to minimize the chromatic aberrations. Careful alignment of the optical elements are performed to achieve optimal two-color volume overlap.

For the dcFCS setup in Gradinaru Lab, the green excitation laser was arbitrarily chosen as the reference path, and was aligned first according to the requirements listed in Section 2.1.4.1. All the detectors and emission pinholes are aligned such that the fluorescent beads show maximum intensity using the green laser. Two-color volume overlap between the green laser and another laser (blue or red) was optimized by adjusting the telescopes and the beam-steering mirrors. In the schematic of the optical layout shown in Figure 2-3 above, lenses $L_{b1}$, $L_{b2}$ form a telescope for the blue laser, $L_{g1}$, $L_{g2}$ for the green laser; and $L_{r1}$, $L_{r2}$ for the red laser. The functions of these telescopes are to re-size the beams and to fine-tune their divergence so that they match each other before the pinhole. Three pairs of mirrors and/or dichroics, i.e., mirrors $M_{b1}$ and $M_{b2}$, mirror $M_{g}$ and dichroic $D_{g}$, mirror $M_{r}$ and dichroic $D_{r}$, are used to independently steer the
corresponding laser beams, such that all lasers are co-centric on the iris located immediately in front of $M$-1.

Taking the two-color alignment between blue and green lasers as an example, the requirements for an optimized two-color alignment are:

1. Both lasers go optimally through the excitation pinhole, the reference irises and the microscope objective.

2. The two confocal images of 100-nm TetraSpeck beads acquired with the two lasers should overlap; center positions of the beads found by 2D-Gaussian fitting should be within 20-nm distance from each other.

3. The divergence of the green beam should be adjusted to minimize the Z-axis offset of the maximal intensity of the two images to within 100 nm.

2.1.4.3 Spectral bleed-through correction and FRET correction

Besides the quality of the alignment, spectral bleed-through and FRET (Förster Resonance Energy Transfer) are two additional effects that can influence the cross-correlation amplitude. Both effects are common between pairs of commercially available fluorophores. Our alignment calibration samples (i.e. the dually labeled DNA for two color volume overlap measurement, see section 2.1.4.4) are not completely free of these artifacts. Consequently, prior to the 2-color volume overlap calibration, a set of well-established procedures, which are described below, needs to be performed to evaluate the spectral bleed through and the FRET contributions to the cross-correlation functions.

Spectral bleed-through, also known as spectral cross-talk, is the phenomenon by which an undesired fraction of the fluorescence signal of a fluorophore is mixed into the detection channel for the other fluorophore. As a result, both of the detection channels will partially contain signals from the same fluorophore, which are always highly correlated. Due to the fact that the cross-correlation function is calculated by correlating the signal from one detection channel to the signal from the other detection channel, the existence of spectral bleed-through will result in false positive cross-correlations.
According to Bacia and co-workers [29], the false positive cross-correlation is an artifact mainly affecting the amplitudes of the correlation functions, without noticeable effect on the diffusion term. Corrections to the spectral bleed-through artifact can therefore be made solely on the fitted initial correlation amplitudes $G_g(0)$, $G_r(0)$, and $G_x(0)$. Furthermore, the emission of the red fluorophore into green detection channel is often negligible. The spectral bleed-through from the green fluorophore to the red detection channel can be corrected by applying the following equations on the fitted correlation amplitudes:

\[
G_g(0) = G_{g,\text{raw}}(0)
\]

\[
G_r(0) = \frac{\kappa^2 f^2 \cdot G_{g,\text{raw}}(0) + G_{r,\text{raw}}(0) - \kappa f \cdot G_{x,\text{raw}}(0)}{(1 - \kappa f)^2}
\]  

(Equation 2.13)

\[
G_x(0) = \frac{G_{x,\text{raw}}(0) - \kappa f \cdot G_{g,\text{raw}}(0)}{1 - \kappa f}
\]

where $G_{i,\text{raw}}(0)$, with $i = g, r, \text{or} x$, are the uncorrected initial correlation amplitudes obtained from fitting the experimental correlation functions. $G_i(0)$, with $i = g, r, \text{or} x$, are the corresponding corrected amplitudes. The factor $\kappa$ is the “bleed-through ratio”. $\kappa$ denotes the ratio of the background-subtracted fluorescence intensity of the green fluorophore in the red channel vs. the green channel.

\[
\kappa = \frac{\langle I_{g,\text{green-only}} \rangle \text{ - bkgd}_g}{\langle I_{g,\text{green-only}} \rangle \text{ - bkgd}_r}
\]  

(Equation 2.14)

In practice, $\kappa$ is determined by measuring a control sample that contains only the green fluorophore. The factor $f$ is the apparent fluorescence intensity ratio. $f$ denotes the ratio of the measured fluorescence intensity in the green channel vs. the intensity in the red channel, for the actual sample that gave rise to the three correlation functions $G_{i,\text{raw}}(\tau)$.

\[
f = \frac{\langle I_g \rangle}{\langle I_r \rangle}
\]  

(Equation 2.15)
Based on Equations 2.13, it can be inferred that lower $\kappa$ and $f$ values are preferred in dcFCS measurements in order to minimize the impact of the correction. Practically speaking, the severity of the spectral bleed-through depends on the choice of fluorophores and spectral filters available, as well as ratio of the abundancy of the two fluorophores. The fluorophores should have emission spectra that are as far-separated as possible. The dichroic and the band-pass filters should block out as much bleed-through signal as possible, while retaining the majority of the desired fluorescence signal. For fluorophores and filters that are used for most of the M$_2$ receptor and G protein project (Figure 2-6), the bleed-through ratio from GFP to mCherry channel with the filter set (Dichroic 585 for splitting signals, then Bandpass 512/25 for GFP and Bandpass 620/52 for mCherry) was determined to be $\kappa \approx 0.04$. Assuming a GFP/mCherry fluorescence intensity ratio of less than 2 ($i.e. f < 2$), the correction needed on the cross-correlation will be less than 10%.

![Figure 2-6](image)

**Figure 2-6.** Transmission spectra of the emission filters used for GFP and mCherry dcFCS measurements, overlaid on the fluorescence spectra of GFP and mCherry. All spectra are normalized to maxima of 1. The spectral bleed-through of GFP fluorescence into the mCherry channel is shaded in light green.

The above-mentioned bleed-through correction method has limitations. Large uncertainties start to appear with $kf > 0.6$. If the product of the bleed-through ratio $\kappa$ and the fluorescence count-rate ratio $f$ from a certain dcFCS measurement is higher than 0.6,
the particular set of dcFCS data should be disregarded. Generally speaking, the bleed-through ratio $\kappa$ can be kept below 0.2 with careful selection of fluorophores and spectral filters. One can also lower the $f$ value of the dcFCS measurements by decreasing the abundance of green-labeled molecules or the excitation laser intensity for the green fluorophore, or increasing the abundance of red-labeled molecules or the excitation laser intensity for the red fluorophore.

Besides the spectral bleed-through, the existence of FRET (Förster Resonant Energy Transfer) is another source of artifacts in dcFCS. Upon a FRET event, a molecule that has both fluorophores bound in close proximity with each other could appear as a red-only molecule. This could negatively affect the measured amplitude of cross-correlation. According to Foo and co-workers, [30] the influence of FRET on the amplitude of the dual-color cross-correlation can be expressed as:

$$G_{x,FRET}(0) = \left( \frac{1 - f_E}{1 - f_E x_g} \right) \left( \frac{1 + q_r}{1 + q_r x_r} \right) \frac{\langle N_g \rangle}{\langle N_g \rangle \langle N_r \rangle}$$

(Equation 2.16)

where $q_i = \eta_{i,FRET}/\eta_{i,noFRET}$ ($i = g$ or $r$) is the ratio of molecular brightness of the donor (or the acceptor) molecule in the presence and in the absence of FRET; $f_E = 1 - q_g$ is the FRET efficiency, assuming a uniform population a single FRET efficiency; $x_i$ ($i = g$ or $r$) is the fraction of green or red molecules that undergo FRET. In the case where all double-labeled molecules have both fluorophores within the Förster Radius, $x_i = frac_i$ ($i = g$ or $r$), and Equation 2.17 can be re-written as:

$$G_{x,FRET}(0) = \left( \frac{1 - f_E}{1 - f_E \cdot f_{cd,g}} \right) \left( \frac{1 + q_r}{1 + q_r \cdot f_{cd,r}} \right) \frac{\langle N_g \rangle}{\langle N_g \rangle \langle N_r \rangle}$$

(Equation 2.17)

Substituting in Equation 2.10, the $f_{cd}$ can be obtained by solving the following equations:
Correcting for FRET artifacts requires separate measurements to determine the FRET efficiency and the molecular brightness of the fluorophores at both non-FRET and FRET situations, as well as the calibration measurement to obtain the OVCF. The FRET correction then involves solving for the roots of a pair of quadratic equations for $f_{cd_g}$ and $f_{cd_r}$. Although this is mathematically manageable, the Equation 2.18 will only hold if the sample displays a single FRET efficiency. In practice, we suggest that the best approach is to choose spectrally well-separated fluorophore pairs and labeling onto non-contacting sites of the two interacting molecules, reducing occurrence of FRET events, thus circumventing the need for FRET correction.

Nonetheless, for a pure double-labeled sample (i.e. $f_{cd_g}=f_{cd_r}=1$), the donor-acceptor cross-correlation will not be influenced by the existence of FRET (Equation 2.18). This property of a pure double-labeled control sample allows for reliable calibration of the two-color detection volume overlap.

2.1.4.4 Design of double-labeled dsDNA

For general applications, the most reliable and practical sample for quantifying and correcting the overlap volume is to use a dual-strand DNA (dsDNA) that is labelled with two different dyes [28]. The cross-correlation amplitude measured on a pure sample of double-labeled dsDNA represents the maximal possible amplitude for optimal alignment, thus providing the foundation for calculating OVCFs as appeared in Equation 2.10.

Several factors need to be taken into consideration when designing an optimal dsDNA oligonucleotide: the choice of fluorophores, the base-pair sequence, and the total number of bases which determines the distance between the probes. The two fluorescent probes should be spectrally well-separated and match the wavelength windows of the detection channels, preferably having similar spectra to, if not the same as, the actual fluorophore
pair used in the dcFCS measurements. Both fluorophores should be labeled on the same strand, leaving the complementary strand unlabeled, so that incomplete hybridization will not affect the correlated behavior of the two fluorophores. The sequence of the base pairs should minimize the occurrence and probability of hairpin and homodimer formation. At the same time, one should avoid placing guanosine (i.e. the G-C pair) immediately next to the fluorophores to prevent quenching [31]. Finally, the total length of the oligonucleotide should be long enough to have negligible FRET efficiency. The long length of the oligonucleotide is a precaution such that even if the DNA molecules are not purely double-labeled, there is still no need to correct for FRET.

For the dcFCS measurements performed throughout this thesis, the optimized dsDNA sequence used for the OVCF calibration measurement is:

5'-(TEX615) TAAGCCTCGTCTGCGTCGGAGCCCGTCTGCCAGCGGAAT (6-FAM) - 3'

with the exact complementary sequence being:

5' - ATTCCGCTGGCAGACGGGCTCCGACGCAGGACGAGGCTT A - 3'

Figure 2-7. Fluorescence spectra comparison of GFP (green), 6-FAM (blue), mCherry (red), and Tex615 (purple). The fluorophores on the dsDNA standard sample (6-FAM and Tex615) are spectrally similar to those of GFP and mCherry.
The oligonucleotide standard samples mentioned above were purchased from Integrated DNA Technologies, Inc. (IDT, [www.idtdna.com](http://www.idtdna.com), USA). The fluorophore pair, 6-FAM (6-Carboxyfluorescein) and Tex615 (a variant of Texas Red), displays similar spectra to those of GFP and mCherry (Figure 2-7). Therefore, the dsDNA can be readily excited with the same blue (488nm) laser and green (532nm) laser, and detected through the same filter sets by the same detectors as in GFP/mCherry measurements. Furthermore, the fluorophores are attached onto the DNA oligonucleotide through phosphoramidite chemistry, ensuring the efficiency and specificity of the labeling. The total length of the oligonucleotide is 40bp, or ~12nm, which is much longer than the Förster radius between 6-FAM and Tex615 (estimated to be at most 5.2nm, assuming a quantum yield of 0.95 for 6-FAM, an extinction coefficient $\varepsilon=85,000\text{M}^{-1}\text{cm}^{-1}$ for Tex615, and a dipole orientation factor $\kappa^2=2/3$ for free rotating fluorophores), thus minimizing the influence of FRET on cross-correlation.

2.1.4.5 Verifying that the dsDNA is fully double-labeled:

The UltraViolet-Visible (UV-VIS) absorption spectroscopy is a widely-used technique specialized in determining the concentration of molecules that contain UV-or-visible-light absorbing π electrons or non-bonding electrons. This method is especially powerful in quantifying protein (mostly with tryptophan or tyrosine absorption, less optimally with peptide-bond absorption), DNA, and fluorescent samples. The concentration of the three components of the calibration DNA sample — nucleotides, 6-FAM, and Tex615 — can be determined using the Beer-Lambert law:

$$\varepsilon = \frac{A}{cl}$$  \hspace{1cm} (Equation 2.19)

where for each absorbing component, $A$ is the measured absorbance at spectral peak, $\varepsilon$ is the extinction coefficient at the wavelength of the spectral peak, and $l$ is the path length of the measurement cuvette.

The UV-Vis results of the dsDNA are shown in Table 2-1. The fact that the estimated concentrations of the DNA oligonucleotide, the 6-FAM dye, and the Tex615 dye are all close to each other is strong evidence for nearly pure double-labelled sample. However,
without further information, one cannot rule out the possibility of an equimolar mixture of free dyes and unlabeled DNA. Such a possibility is rejected because the FCS curve of the dsDNA was explained well with one diffusing component (Figure 2-8); and no physically relevant fast-diffusing component could be retrieved when fitting the FCS curve to a two-component diffusion model (data not shown).

**Table 2-1.** A representative UV-Vis result of a dual color calibration DNA sample. The measurement was taken on a ThermoFisher NanoDrop 1000 Spectrophotometer. The path length \( l \) of the setup is fixed at 0.1 cm.

<table>
<thead>
<tr>
<th></th>
<th>Max extinction wavelength ( \lambda ) (nm)</th>
<th>Max Extinction coefficient ( \varepsilon ) *1 ((\text{L/mol/cm}))</th>
<th>Absorbance ( A ) (OD)</th>
<th>Concentration *2 ((\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (40bp)</td>
<td>260</td>
<td>395,700</td>
<td>0.696</td>
<td>17.6</td>
</tr>
<tr>
<td>6-FAM</td>
<td>495</td>
<td>75,000</td>
<td>0.129</td>
<td>17.2</td>
</tr>
<tr>
<td>Tex615</td>
<td>615</td>
<td>85,000</td>
<td>0.142</td>
<td>16.7</td>
</tr>
</tbody>
</table>

*1: The extinction coefficient of the DNA backbone also includes the contribution of the fluorophores. *2: The concentration values were estimated using Equation 2.19.

### 2.1.4.6 Procedures for calculating the overlap volume correction factors

Just as other double-labeled samples, the FAM-dsDNA-TEX sample has some level of spectral bleed-through that affects the dcFCS measurements. The bleed-through ratio \( \kappa \) needs to be quantified prior to the calculation of OVCFs. Ideally, the best sample to quantify it would be a sample of 6-FAM-dsDNA. However, in practice a sample of free fluorescein dye was used. This is justified because the emission spectrum of the free fluorescein and that of the 6-FAM on double-labeled dsDNA were found to be nearly identical in TE buffer (10mM Tris, 1mM EDTA, adjusted to pH 8). The bleed-through ratio \( \kappa \) was determined using the free fluorescein on the dcFCS microscope with all relevant spectral filters applied, by simply taking the ratio of fluorescence signals between the red channel and the green channel according to Equation 2.8.

Once the bleed-through ratio \( \kappa \) is determined, the dcFCS calibration measurement on the FAM-dsDNA-TEX sample can be performed on the properly aligned setup. The resultant
dcFCS curves, including two auto-correlation functions and one cross-correlation function, are fitted to appropriate models to retrieve the raw correlation amplitudes \( G_{g,\text{raw}}, G_{r,\text{raw}}, \) and \( G_{x,\text{raw}}. \) Since the true fraction of co-diffusing species is \( \sim 100\% \) according to the quality-control experiments, the difference between the raw cross-correlation and auto-correlation functions is caused primarily by the two-color volume misoverlap and by spectral bleed-through.

The bleed-through artifact can be removed from the correlation functions using the fluorescence signal intensities acquired during the dsDNA measurement. The removal procedure follows Equation 2.13, in which the bleed-through ratio \( \kappa \) is obtained from the single fluorescein measurement, and the fluorescence count-rate ratio, \( f \), is calculated from the dsDNA dcFCS measurement by taking the ratio between the fluorescence intensities of FAM and Tex615.

We define the correlation amplitudes after the bleed-through correction as \( G_{g}, G_{r}, \) and \( G_{x}. \) (the green auto-correlation amplitude remains the same as its raw value \( G_{g,\text{raw}}. \)) According to Eq. 2.10, when the \( fcd_{g} \) and \( fcd_{r} \) are known to be 1, a pair of overlap volume correction factors (OVCF) for the auto-correlation functions can be defined from the amplitudes of the dsDNA measurement:

\[
\begin{align*}
\text{OVCF}_{g} & \equiv \left( \frac{V_{\text{eff},g}}{V_{\text{eff},x}} \right) = \frac{G_{r,\text{dsDNA}}(0)}{G_{x,\text{dsDNA}}(0)} \cdot \frac{c_{x,\text{dsDNA}}}{c_{g,\text{dsDNA}}} = \frac{G_{r,\text{dsDNA}}(0)}{G_{x,\text{dsDNA}}(0)}, \\
\text{OVCF}_{r} & \equiv \left( \frac{V_{\text{eff},r}}{V_{\text{eff},x}} \right) = \frac{G_{g,\text{dsDNA}}(0)}{G_{x,\text{dsDNA}}(0)} \cdot \frac{c_{x,\text{dsDNA}}}{c_{r,\text{dsDNA}}} = \frac{G_{g,\text{dsDNA}}(0)}{G_{x,\text{dsDNA}}(0)).
\end{align*}
\]

(Equation 2.20)

Note that for the pure double-labeled dsDNA, \( c_{d}/c_{g} = c_{d}/c_{r} = 1. \) All subsequent dcFCS measurements taken under the same alignment conditions will have the raw amplitudes adjusted, first by the bleed-through corrections and then by the overlap volume correction factors.
Figure 2-8. dcFCS curves measured on a FAM-dsDNA-TEX calibration sample. The intensity traces from the 4 channels are shown in the inset for determination of the apparent fluorescence intensity ratio \( f \) that is needed for spectral bleed-through correction.

As an example, the full process for obtaining the overlap volume correction factors for the measurement shown in Figure 2-8 proceeds as follows:

**Step 1.** In the fluorescein dye measurement, the fluorescence count-rate in the green and the red channels are 89.2kcps and 8.02kcps respectively. The bleed-through ratio \( \kappa \) is calculated as \( \kappa = \frac{7.72}{88.9} = 0.087 \), where the background count rate of 0.3kcps has been subtracted from both channels.

**Step 2.** In the dsDNA measurement, the fluorescence count rate in the green and the red channels are 18.6kcps and 16.2kcps respectively. The apparent fluorescence intensity ratio \( f \) is calculated as \( f = \frac{18.6}{16.2} = 1.15 \). Now we have the product \( \kappa f = 0.10 \).

**Step 3.** The raw uncorrected amplitudes of the three raw correlation functions from the dsDNA calibration measurement are obtained by fitting the curves to the (3D 1-component-normal-diffusion-1-triplet) FCS model: \( G_{g,\text{raw}}(0) = 0.156; G_{r,\text{raw}}(0) = 0.206; G_{x,\text{raw}}(0) = 0.110 \).
**Step 4.** Following Equation 2.13, the raw red auto-correlation and raw cross-correlation amplitudes from **Step 1** are corrected for spectral bleed-through as

\[
G_{g}(0) = G_{g,\text{raw}}(0) = 0.156 \\
G_{r}(0) = \frac{\kappa^2 f^2 \cdot G_{g,\text{raw}}(0) + G_{r,\text{raw}}(0) - \kappa f \cdot G_{s,\text{raw}}(0)}{(1-\kappa f)^2} = \frac{0.01 \times 0.156 + 0.206 - 0.1 \times 0.110}{(1-0.1)^2} = 0.242 \\
G_{x}(0) = \frac{G_{x,\text{raw}}(0) - \kappa f \cdot G_{g,\text{raw}}(0)}{1 - \kappa f} = \frac{0.110 - 0.1 \times 0.156}{1 - 0.1} = 0.105
\]

**Step 5.** The overlap volume correction factors are calculated using Equation 2.20 as

\[
\text{OVCF}_g = \frac{G_{r,\text{dsDNA}}(0)}{G_{x,\text{dsDNA}}(0)} = \frac{0.242}{0.105} = 2.30; \\
\text{OVCF}_r = \frac{G_{g,\text{dsDNA}}(0)}{G_{x,\text{dsDNA}}(0)} = \frac{0.156}{0.105} = 1.49
\]

These correction factors will be applied to all the subsequent dcFCS measurements taken under the same alignment (using Equation 2.10) to estimate the true fractions of co-diffusion (fcd).

2.1.4.7 Negative control sample: a mixture of free dyes

In addition to the double-labeled dsDNA control, we also tested a mixture of free fluorescein and Texas Red dyes. In theory, for the independent dye molecules, zero fractions of co-diffusion are expected. Any positive cross-correlation amplitude should be the result of spectral bleed-through artifact.
Figure 2-9. Raw dcFCS curves from a mixture of free fluorescein and Texas Red dyes and the corresponding intensity traces. The low cross-correlation between independently diffusing molecules serves as a verification that no significant false-positive artifacts are induced.

For the measurement shown in Figure 2-9, the raw amplitudes of the correlation curves were $G_{g,raw}(0)=0.0221$, $G_{r,raw}(0)=0.0495$, and $G_{x,raw}(0)=0.00262$ as retrieved from fitting the curves to the (3D 1-component-normal-diffusion-1-triplet) FCS model. The bleed-through ratio $\kappa$ was previously determined in section 0 for fluorescein: $\kappa=0.087$. The apparent fluorescence intensity ratio $f$ was calculated from the fluorescence intensity traces: 

$$f = \frac{18.2kHz + 16.1kHz}{14.8kHz + 13.3kHz} = 1.22$$

Therefore $\kappa f = 0.106$, and the correlation amplitudes after the correction for spectral bleed-through can be calculated according to Equation 2.13:
\[ G_g(0) = G_{g,\text{raw}}(0) = 0.0221 \]
\[ G_r(0) = \frac{\kappa f^2 \cdot G_{g,\text{raw}}(0) + G_{r,\text{raw}}(0) - \kappa f \cdot G_{s,\text{raw}}(0)}{(1 - \kappa f)^2} = \frac{0.01 \times 0.0221 + 0.0495 - 0.106 \times 0.00262}{(1 - 0.106)^2} = 0.0617 \]
\[ G_s(0) = \frac{G_{s,\text{raw}}(0) - \kappa f \cdot G_{g,\text{raw}}(0)}{1 - \kappa f} = \frac{0.00262 - 0.106 \times 0.0221}{1 - 0.106} = 3.58 \times 10^{-7} \]

Hence the fraction of co-diffusion can be determined according to Equation 2.10:

\[ f_{cd_g} = \frac{G_r(0) \cdot \text{OVCF}_g}{G_g(0)} = \frac{3.58 \times 10^{-7}}{0.0617} \times 2.30 = 1.33 \times 10^{-5}; \]
\[ f_{cd_r} = \frac{G_s(0) \cdot \text{OVCF}_r}{G_g(0)} = \frac{3.58 \times 10^{-7}}{0.0221} \times 1.49 = 2.43 \times 10^{-5}. \]

In contrast, the apparent fractions of co-diffusion without the correction for spectral bleed-through would be:

\[ f_{cd_g,\text{raw}} = \frac{G_{s,\text{raw}}(0)}{G_{r,\text{raw}}(0)} \cdot \text{OVCF}_g = \frac{0.00262}{0.0495} \times 2.30 = 0.121; \]
\[ f_{cd_r,\text{raw}} = \frac{G_{s,\text{raw}}(0)}{G_{g,\text{raw}}(0)} \cdot \text{OVCF}_r = \frac{0.00262}{0.0221} \times 1.49 = 0.178. \]

It can be seen that the correction method described in Sections 2.1.4.3 and 2.1.4.6 effectively removed almost all the artifact caused by spectral bleed-through.

### 2.2 Two-Photon Excitation FCS Technique

Two photon excitation is a nonlinear process in which a molecule absorbs two photons of identical or different frequencies within 1 fs \((10^{-15} \text{ s})\) [32] and then makes a transition to a higher energy state (Figure 2-10). The energy difference between the involved lower and upper energy states of the molecule is equal to the sum of the photon energies of the two photons. In the case of a fluorescent molecule, the electronic state induced by two photon absorption will also result in an emission photon with the same spectrum as that from a one photon excitation (OPE) event (Figure 2-10) [33]. During the TPE process, the transition probability to the excited state is proportional to the square of the incoming...
laser intensity (therefore it is a second-order nonlinear process) [32]. In a confocal TPE microscope with a high numerical aperture objective, the excitation laser intensity approximately forms a 3D Gaussian distributed focal volume, and the fluorescence excitation is limited to the focal volume. Consequently, the TPE microscope shows a number of advantages over the familiar OPE such as well-separated excitation and emission wavelengths, higher axial resolution, increased penetration depth, reduced out-of-focus noise, and lower chances of photobleaching and photodamage.

Figure 2-10. Jablonski diagrams for the OPE (left) and TPE (right) processes of a fluorophore. In OPE, fluorophores excited to the excited state by absorption of a single photon of a specified energy (colored blue) can result in the emission of a photon of lower energy (colored green). In TPE where two photons, each with half the required energy (colored red), are absorbed simultaneously, the emitted photon can have higher energy (colored green) than the excitation photons. (Image credit: Durst Research Group, Middlebury College, USA.)

The femtosecond pulsed laser in Gradinaru lab (Tsunami HP Ti-Sapphire laser system, Spectra Physics, Santa Clara, CA, USA) was routed to the dcFCS microscope for TPE measurements. When mode-locked, the Tsunami laser outputs a train of femtosecond pulses with a center wavelength tunable from 700 nm to 1100 nm, allowing for exciting a wide range of commonly used fluorescent dyes [33, 34] and fluorescent proteins [35]. The duration of each pulse is typically ~80 fs (at 960nm wavelength), containing up to
5×10^9 photons per pulse passing through the detection volume (at the maximum power setting of 50mW at the objective), achieving much higher TPE efficiency than picosecond lasers.

---

**Figure 2-11.** The quadratic relation between 2PE fluorescence intensity, \( I \) (i.e. number of photons received by the detector per second, unit: kHz), and power of the laser at the objective, \( P \) (unit: mW). The fitted quadratic function is \( I = 14.9 - 4.58P + 1.47P^2 \). The constant term (14.9 kHz) denotes the power-independent background noise; the linear term (-4.58 kHz/mW) denotes a decreasing trend of OPE contribution to the total fluorescence intensity; the significant quadratic term (1.47 kHz/mW^2) is a characteristic feature of TPE process.

As an initial test, a 20nM Rhodamine110 (R110) solution was measured with the Tsunami laser mode-locked at 960nm. The underlying TPE process was verified by the quadratic dependence [36] of fluorescence emission intensity on the excitation intensity (Figure 2-11). FCS curves were collected and fitted to the same 1-color FCS model for OPE (Equation 2.3) [37] to find the diffusion time \( \tau_D \). In order to calculate the dimensions of the 3D Gaussian TPE detection volume \( w_0 \) and \( z_0 \), the following equation is used to interpret the \( \tau_D \) of the calibration dye R110 [38]:

\[
w_0^2 = 8D\tau_D
\]
where the multiplier ‘8’ is different from the value ‘4’ in OPE, because the intensity profile is squared to provide the two-photon excitation profile [7, 39]. From four FCS measurements of the same R110 sample on four different alignments, the $w_0$ and $z_0$ parameters were found to be stable and consistent, with values $w_0 = 293 \pm 24 \text{nm}$, and $z_0 = 1.9 \pm 0.3 \mu m$.

![Figure 2-12. Raw 2PE-dcFCS measurement on double-labeled dsDNA. After correcting for spectral bleed-through, OVCF$_{g}$ and OVCF$_{r}$ were determined to be 1.17 and 1.37, respectively.](image)

If both fluorophores involved in a dcFCS measurement can be excited with the same laser through TPE process, the TPE-dcFCS eliminated the need to optimize or account for the overlap of the excitation volumes. In fact, most fluorophores exhibit wider excitation spectra in TPE process compared to OPE [33-35, 40], bringing a key advantage to TPE-dcFCS over OPE-dcFCS.

The two fluorophores (6-FAM and Tex615) on the double-labeled dsDNA designed in Section 2.1.4.5 are able to be excited simultaneously with TPE at 960nm. The identical excitation volumes for both fluorophores were verified with dcFCS measurements on the dsDNA with the Tsunami laser at 960nm. The OVCF’s were determined to be 1.17 for green excitation volume and 1.37 for red excitation volume (Figure 2-12), noticeably closer to 1 than the values obtained from OPE-dcFCS in Section 0 (2.30 for green and
1.49 for red). The deviation from the theoretical value of 1 could be the result of a series of factors such as uncorrelated background signal or inactive fluorophores. [41]

The TPE FCS is promising technique for studying the membrane receptors in live cells [42]. Because TPE was a recent development on the dcFCS microscope in the Gradinaru Lab, most of the dcFCS measurements reported in this thesis were performed with OPE, and therefore required corrections for the overlap of excitation volumes. Future dcFCS measurements can receive the benefit of the TPE technique provided that the two fluorophores can be excited simultaneously with a single wavelength.

2.3 SMF Microscopy Techniques

Confocal fluorescence spectroscopy have proven to be exceptionally insightful in probing parameters related to the conformation, motion, and functional interaction of biomacromolecules [43-46]. However, techniques of spectroscopy fall short of techniques of microscopy by the richness and authenticity of the visual information. More specifically, the FCS technique draws conclusion from all the molecules that diffuse through the detection volume over the course of the measurement, which renders the method inherently incapable of resolving the hidden heterogeneity of individual molecules. To supplement the FCS measurements and study the sub-ensemble behaviors of the receptors and G proteins, single molecule fluorescence (SMF) microscopy was needed.

The chief technical difficulty in SMF microscopy is the conflict between sensitivity, speed and background noise. The detection sensitivity has to be high enough to register the weak fluorescence signals from single fluorophores, while at the same time, the noise from the sample environment and camera readout process must not overwhelm the SMF signal. Furthermore, the laser excitation power should be limited to prevent rapid photobleaching of the fluorophores. These constraints amount to serious challenges for both microscope performance and sample experimental conditions. Among a handful of microscopy techniques that satisfy the SMF constraints, the Total Internal Reflection Fluorescence Microscopy is one of the most fruitful techniques [6, 47-49] that excels for both functionally purified samples and live-cell samples.
2.3.1 The TIRF microscope

In the Gradinaru lab, Total Internal Reflection Fluorescence (TIRF) microscopy is employed for real-time visualization of fluorescent-labeled single molecules. Utilizing the evanescent field created by total internal reflection and an objective-based inverted TIRF microscope, one is capable of selectively illuminating the sample molecules above the water-coverglass interface within 50~300 nm penetration depth. This thin layer of illumination greatly increases the signal-to-noise ratio by discarding most of the fluorescence background originating from deeper sections of sample solution.

The desired detection efficiency and signal-noise ratio are achieved in the setup with the help of the back-illuminated Andor EMCCD camera (iXon DU-897BV). The camera has several outstanding advantages such as 33 full frames (512x512 pixels) per second acquisition rate, ~90% quantum detection efficiency in the visible wavelength range, less than single-electron readout noise and user-adjustable electron multiplier gain, making it ideal for SMF applications. When running in cropped/binned mode, the acquisition rate of the camera can be pushed up to 439 frames per second (with 4x4 binning and 128x128 pixels per frame), or 2.3ms per frame, allowing for the capture of a wide range of physiological processes (such as diffusion of biomolecules in cells) and photophysical processes (such as photobleaching of the fluorophores).

Other convenient features of the setup include: four independent laser lines (405nm, 473nm, 532nm, 633nm) for multi-color imaging or alternating laser excitation imaging; an acousto-optic tunable filter (AOTF) for fast laser excitation switching/mixing; motorized incident angle control stage for adjusting the TIR penetration depth; piezo objective mount for fine tuning of the focus of imaging; motorized X-Y sample stage; signal splitting options for either color-based dual channel imaging with emission dichroics, or polarization-based dual channel imaging with polarizing cubes; and a heated stage-top incubator chamber for live cell imaging. The optical layout of the TIRF Microscope setup is shown in Figure 2-13.
Figure 2-13. Schematic optical layout of the TIRF Microscope in Gradinaru Lab (courtesy of Zhenfu Zhang). The microscope is capable of 4-color alternating laser excitation and dual color/polarization channel SMF measurements.

Two types of SMF microscopy experiments are conducted on the TIRF microscope throughout this thesis, namely the single-molecule photobleaching (smPB) measurements and the single-particle tracking (SPT) measurements.

2.3.2 Single-molecule photobleaching (smPB) measurements

Combined with single-molecule immobilization methods [50, 51], the TIRF microscope is employed for single-molecule photobleaching analysis of the oligomeric sizes of biomacromolecules. The sample molecules are uniformly labeled with exactly one fluorophore per protomer, and then immobilized onto the activated surface of coverslip. Each single molecule then appears as a single dot with diffraction limited size under the TIRF microscope, with the individual molecular brightness proportional to its oligomeric size. The excitation laser is applied continuously, thus the molecular brightness for all the molecules will drop in a step-wise manner as fluorophores become photobleached. The number of photobleaching steps per molecule is combined into a histogram to reveal the distribution the oligomeric size of the sample (Figure 2-14). [52]
Figure 2-14. A smPB experiment of multi-step constructed DNA ladder molecules immobilized on a BSA-biotin activated surface. (A) TIRF movie was recorded until all the immobilized molecules are photobleached. (B) Step-wise photobleaching behavior was observed. For each molecule, the number of photobleaching steps was extracted from the intensity-time (I-t) traces with change-point analysis. (C) The histogram of the number of photobleaching steps per molecule was interpreted as the distribution of oligomeric size. This DNA ladder sample was determined to consist of a majority of small oligomers with sizes less than 4 and a minority of large oligomers with sizes up to 12.

The single molecule photobleaching technique is extensively applied in Chapter 4, “smPB studies of the oligomeric status of M₂ receptors and G proteins in their signaling complex”.

2.3.3 Single-particle tracking (SPT) measurements

Spatio-temporal tracking of single molecules was carried out in live cells on the TIRF Microscope to study the distribution, diffusion, and interaction of fluorescently-labeled proteins in live cells. Typically, TIRF movies of fluorescently labeled proteins diffusing at the bottom membrane of the cells (and thus within the TIR evanescent illumination field) are analyzed with automated programs carrying out a multi-particle tracking algorithm [53, 54]. The detected trajectories of the tracked particles can be further analyzed to measure the diffusion properties, dynamic oligomerization, and complex formation of the receptors and G proteins in their native environment (Figure 2-15).
Figure 2.15. Quasi single-molecule tracking results of M₂-GFP in a live CHO cell. (A) TIRF image sequence of the GFP-labeled M₂ receptors in a live CHO cell. (B) Particle identified by the TrackMate software, shown as cyan circles overlay. (C) The tracking trajectories output by the TrackMate software, color coded in temporal order (early trajectories in blue, late trajectories in red).

The single molecule tracking technique is extensively applied in Chapter 5. “Diffusion behaviors and oligomeric size of M₂ receptors and G proteins observed in live cells by single particle tracking.”

2.4 References


3 DcFCS on Receptor-G Protein Complex

3.1 Introduction and Motivation

As the largest family of membrane signal receptors, the GPCRs are truly outstanding in their signal transduction efficiency. Their efficiency manifests itself in two different ways: speed and sensitivity. For instance, the rhodopsin receptor in the mammalian visual system has the ultimate single-photon detection sensitivity [1-4]. It has been reported that the total time it takes between the binding of an external signal ligand to the receptor and the activation of effector proteins is less than 1 second for most of the commonly studied GPCR subtypes [5-8]. As for the M$_2$ muscarinic receptor, the binding of an agonist to the receptor initiates downstream signaling via interactions with its specific G protein (G$_{i1}$). The downstream activity of the muscarinic receptors has been detected within 300ms after stimulation [9, 10]. Therefore, understanding the activation process and the factors that influence the activation is a key point of interest in the research of GPCR.

The coupling mechanism between a receptor and its attendant G protein dictates the rate and efficiency of GPCR-mediated signaling processes [11, 12]. The complex of receptor and G protein could either be stably formed prior to the ligand binding to the receptor or transiently formed due to stochastic interactions. The fast signaling process could result either from the conformational changes within a preformed receptor-G protein complex or from a proximity-induced, transient coupling between a receptor and a G protein [13]. Obtaining a mechanistic picture of the receptor signaling cycle thus relies on differentiating between the two scenarios.

The question whether GPCRs exist and function, at least in part, as homo- or hetero-oligomeric complexes has also been a topic of much debate [14, 15]. Some evidence points to monomers [16, 17], while other studies invoke the existence of homodimers [18-20], heterodimers [21, 22], or higher order oligomers [23, 24] of receptors. Oligomeric receptors and their cooperative behaviors could provide new insights into the mechanism of receptor signaling pathways, and open up possibilities for new targets as it is related to therapeutic intervention [24].
The advantage of dcFCS over other fluorescence spectroscopy and imaging techniques in studying protein complexes is that co-diffusion provides an unambiguous indicator of coupling [25]. On the other hand, dcFCS, while sensitive to the presence of oligomeric complexes and providing the fraction of proteins forming oligomers, has limitations in quantifying the size of those oligomers. This is done by single-molecule photobleaching (smPB) stoichiometry, with the results summarized in Chapter 4.

### 3.2 Materials and Methods

#### 3.2.1 Sample preparation

For dcFCS experiments, the M₂ receptors and the Gᵢ₁ proteins were co-expressed with GFP and mCherry. As discussed in Chapter 2.1.4.3, before the cross-correlation measurements, the contribution of spectral bleed-through was quantified using GFP-only samples. In the case of co-expression, to keep the false positive cross-correlation amplitude below 5%, the transfection quantity and therefore the expression level of mCherry-labeled molecules was deliberately increased to ~1.5 times that of the GFP-labeled molecules.

#### 3.2.1.1 Measurements in detergent micelles: protein expression and purification

The sample preparation for the measurements in detergent micelles was conducted in the Wells Lab following the protocol below.

Baculoviruses coding for GFP- or mCherry-bearing human M₂ muscarinic receptor and for GFP- or mCherry-inserted αᵢ₁, wild-type β₂, and wild-type γ₁ subunits of the G protein were expressed in Sf9 cells. The M₂ receptor and Gαᵢ₁ subunit are fused with His₆ polyhistidine tag to facilitate purification. Sf9 cells were prepared as described previously [26]. The cells were cultured at 27 °C in Ex-Cell 400 insect media (JRH Biosciences) containing fetal bovine serum albumin (2%), Fungizone (1%), and gentamycin (0.1%) (Life Technologies, Gibco-BRL) and grown to confluence at a density of 2 × 10⁶ cell/ml. When in the exponential phase of growth, the cells were co-infected with baculoviruses encoding the subject proteins at a total multiplicity of infection of five plaque-forming
units per cell. They were harvested 48 h after infection, collected by centrifugation for 15 min at 4 °C and 1,000 × g, and then either immediately extracted for protein or temporarily stored at −75 °C.

**Extraction of isolated receptor and isolated G protein.** The isolated receptor and isolated G protein purification procedures used in this project were adopted from [27, 28]. For extracting the receptor, the harvested Sf9 cells were homogenized with three 15sec sonication pulses of a Bio-Homogenizer (Biospec Products Inc.). The homogenate was washed twice in buffer D (20 mM KH₂PO₄, 20 mM NaCl, 1 mM EDTA (Ethylenediaminetetraacetic acid), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 tablet of cOmplete Protease Inhibitor Cocktail (Sigma-Aldrich), adjusted to pH 7.4 with NaOH) by centrifugation for 45 min at 4 °C and 100,000 × g. The receptor was extracted from the washed membranes in 0.89%-0.16% digitonin–cholate (Wako Chemicals Inc.). The solubilized product would be passed through nickel-nitrilotriacetic acid (Ni²⁺-NTA) agarose column (Qiagen #30210) for purification. For extracting the G protein, the harvested sf9 cell pellets were homogenized with three sonication pulses of a Bio-Homogenizer. The homogenate was washed twice in buffer A (20 mM KH₂PO₄, 20 mM NaCl, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.1 mM EDTA, 10 mM β-mercaptoethanol, 1 tablet of cOmplete Protease Inhibitor Cocktail, adjusted to pH 7.4 with NaOH) by centrifugation for 30 min at 4 °C and 100,000 g. The washed membranes were re-suspended with 1% (w/v) sodium cholate (Sigma-Aldrich) at 4 °C overnight. The suspended product would be passed through Ni²⁺-NTA agarose column for purification.

**Purification of isolated receptor and isolated G protein.** The solubilized receptor and the suspended G protein were purified by passing through the Ni²⁺-NTA column. Prior to loading the sample, the column was equilibrated with equilibrating buffer Qₚ (for receptor, 20 mM HEPES, 20 mM NaCl, 1 mM MgCl₂ (Sigma-Aldrich), 10 mM β-mercaptoethanol, 0.22%-0.04% digitonin-cholate, adjusted to pH 7.4 with NaOH) or equilibrating buffer Q₇ (for G protein, 20 mM HEPES, 20 mM NaCl, 1 mM MgCl₂ (Sigma-Aldrich), 10 mM β-mercaptoethanol, and 0.2% sodium cholate, adjusted to pH 7.4 with NaOH). After loading the sample, the column was washed two column volumes
of wash buffer $W_R$ (for receptor, 20mM HEPES, 3mM MgCl$_2$, 10mM $\beta$-mercaptoethanol, 60mM imidazole, 100 mM NaCl, 0.22%-0.04% digitonin-cholate) or wash buffer $W_G$ (for G protein, 20mM HEPES, 3mM MgCl$_2$, 10mM $\beta$-mercaptoethanol, 60mM imidazole, 100 mM NaCl, 0.2% sodium cholate) to remove non-specifically bound impurities. Finally the His$_6$-tagged receptors or His$_6$-tagged G proteins were retrieved from the column with elution buffer $E_R$ (for receptor, 20mM HEPES, 20mM NaCl, 3mM MgCl$_2$, 10mM $\beta$-mercaptoethanol, 150mM imidazole, 0.86%–0.17% digitonin-cholate, adjusted to pH 7.4 with NaOH) or elution buffer $E_G$ (for G protein, 20mM HEPES, 20mM NaCl, 3mM MgCl$_2$, 10mM $\beta$-mercaptoethanol, 150mM imidazole, 1% sodium cholate, adjusted to pH 7.4 with NaOH). The purified sample was then stored on ice and ready for measurement.

**Extraction of coupled receptor and G protein.** Extraction of the samples containing both receptor and G protein followed steps similar to those described above for the isolated receptor. The key additions to the co-purification procedure included the incorporation of the agonist carbachol and the steps to remove bound GDP for the isolation of the complex. The co-infected Sf9 cell pellets were lysed with buffer $A_{RG}$ (10 mM carbachol, 20 mM KH$_2$PO$_4$, 20 mM NaCl, 20 mM HEPES, 0.1 mM EDTA, 10 mM $\beta$-mercaptoethanol, Complete Protease Inhibitor tablets, and 200 $\mu$g/mL bacitracin (Sigma-Aldrich), adjusted to pH 7.4 with NaOH). After centrifugation, the pellets were washed three times with buffer $B_{RG}$ (i.e. buffer $A_{RG}$ supplemented with 1 mM EDTA to remove GDP). The membrane was subsequently washed with buffer $C_{RG}$ (i.e. $A_{RG}$ without EDTA). The washed membrane was then re-suspended in buffer $C_{RG}$, and 25 mU/mL apyrase (New England Biolabs) was added to the homogenate. The mixture was inverted gently at 4 °C for 60 min so that bound GDP was hydrolyzed by the purine pyrophosphatase apyrase. (The GDP removal are crucial to the isolation of a coupled receptor-G protein complex as the GDP-bound G protein disrupts the stability of the receptor-G protein complex.) Finally, the membrane was resuspended to approximately 5.5 mg/mL protein and solubilized in digitonin–cholate (0.86% digitonin, 0.17% cholate) in the presence of 10 mM carbachol before purification on the Ni$^{2+}$-NTA column.
Purification of coupled receptor and G protein. Purification of the receptor-G protein complex was similar to that of the isolated receptor, with minor alternations to the buffers. Prior to loading the sample, the Ni$^{2+}$-NTA column was equilibrated with the equilibration buffer $Q_{RG}$ (20 mM HEPES, 20 mM KH$_2$PO$_4$, 20 mM NaCl, 10 mM β-mercaptoethanol, 10 mM carbachol, and 0.22%-0.04% digitonin–cholate). After loading the sample, the column was washed with two volumes of wash buffer $W_{RG}$ (20 mM HEPES, 20 mM KH$_2$PO$_4$, 10 mM β-mercaptoethanol, 10 mM carbachol, 60 mM imidazole, 0.22%-0.04% digitonin–cholate, and 100 mM NaCl). The His$_6$-tagged receptor-G protein complex was then retrieved from the column with the elution buffer $E_{RG}$ (20 mM HEPES, 20 mM KH$_2$PO$_4$, 20 mM NaCl, 10 mM β-mercaptoethanol, 10 mM carbachol, 150 mM imidazole, and 0.86%-0.17% digitonin–cholate, adjusted to pH 7.4 with NaOH). The purified sample was then stored on ice and ready for measurement.

3.2.1.2 Measurements in live cells: CHO cell cultures

Chinese Hamster Ovary - S (CHO-S) cells were chosen as the host cell line for dcFCS experiments *ex vivo*. CHO cells are an epithelial cell line derived from the ovary of the Chinese hamster, which has proven to be a reliable cell line for biomolecular research on mammalian recombinant proteins. The CHO-S cell line that was used in our studies was purchased from ThermoFisher as part of the *FreeStyle MAX Expression System*.

CHO cells were grown in Dulbecco's modified Eagle's medium containing 4.5g/L glucose and L-glutamine (Corning Cellgro) supplemented with 10 % (v/v) fetal bovine serum (Gibco), 0.1 mM non-essential amino acids (Gibco), 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich). The cell line was grown in a 30-mm diameter disposable Petri dish. The cells were split 1:10 when the confluency reached above 80%. Upon splitting, part of the cells were plated on glass-bottom dishes to prepare for transfection and observation.

The cells were transfected at approximately 30–40 % confluency using GeneExpresso Max Transfection Reagent (Excellgen, USA). The DNA and the transfection reagent were used at a ratio of 1:3 as instructed by the manual provided by the manufacturer. The total amount of DNA used was in a range of 0.5–2 μg. The cells were ready for
measurement within a 24–48 hours window after transfection. Immediately prior to measurement, the cell culture media was changed to imaging medium (CHO Minimum Essential Medium containing 4.5 g/L glucose, L-glutamine, and supplemented by 25mM HEPES). The supplemented HEPES maintains the pH of the medium within the optimal physiological range 7.0–7.6 at room temperature 25 °C under regular atmospheric conditions (i.e. without heating or CO₂ supply).

3.2.1.3 Quantification of endogenous G proteins

M₂ receptors are not natively present in CHO cells [29] or Sf9 cells [30]. The measurements on the transfected or infected M₂ receptors are therefore not likely influenced by the endogenous receptors.

G proteins, on the other hand, are widely involved in the signaling mechanisms of most eukaryotic cells. Using Western blot with anti-Gα₁, we compared the quantity of the endogenous G proteins with the transfected G proteins in CHO cells and the infected G proteins in Sf9 cells. As shown in Figure 3-1, the quantities of endogenous G proteins in both CHO and Sf9 cells were negligible relative to the exogenous G proteins. Similar Western blot results were observed on two other batches of G proteins extracts. Hence, the influence of unlabeled G proteins on the fluorescence spectroscopy measurements should be limited.

![Western blot Figure](image.png)

**Figure 3-1.** Solubilized extracts from cells expressing only endogenous G protein (lanes 1 and 3) and cells transfected or infected with His₆-Gα₁ subunits (lanes 2 and 4) were blotted with anti-
Gαi1. Lanes 1 and 2 were from CHO cells (22 μg of protein/lane); lanes 3 and 4 were from Sf9 cells (72 μg of protein/lane). The transfection of CHO cells and infection of Sf9 cells were described in Sections 3.2.1.1 and 3.2.1.2.

3.2.2 DcFCS Experimental Setup

All of the detergent-solubilized and most of the live-cell dcFCS measurements were performed on the custom-built dcFCS microscope in the Gradinaru Lab. The detailed description of the dcFCS microscope can be found in Chapter 2.1.3. In brief, a confocal microscope was built in the Gradinaru Lab with multi-color detection and real-time multi-channel correlation analysis capability. To excite the GFP and mCh simultaneously for the current research, a blue 488nm laser (TECBL-488nm, WorldStarTech) and a green 532nm laser (MatchBox2-532nm, IntegratedOptics) were used. Excitation power at the objective was kept between 0.1–5 μW, corresponding to 0.05–2.5 kW/cm² intensity at the diffraction-limited focal spot. The GFP and mCh fluorescence signals were split by a 585nm emission dichroic (FF585-Di01, Semrock). The GFP signal was further filtered by a 512/25 nm bandpass filter (FF01-512/25, Semrock), whereas the mCh signal was further filtered by a 620/52 nm bandpass filter (FF01-620/52). Four avalanche photodiode (APD) detectors (SPCM-AQR-13-FC, PerkinElmer Inc.) were employed (two each for GFP channel and mCh channel, respectively) to collect fluorescence signals from the sample. Correlation curves were retrieved in real-time from the hardware correlator (Flex11-8Ch, correlator.com). For a sample solubilized in detergent micelles, more than $10^7$ photons were typically collected at a count rate of 5–20kHz. For a sample in live cells, $0.5–5\times10^6$ photons were typically collected at a count rate of 20–100kHz.

Additional live cell dcFCS measurements of various samples were performed on a Nikon Eclipse Ti inverted microscope system (The Imaging Facility at Hospital for Sick Children, Toronto) for comparing the experimental conditions. The Nikon microscope is equipped with an on-stage heated CO₂ chamber that is beneficial for keeping the cells in their growth conditions. However, the dcFCS setup in the Gradinaru Lab is preferred for its specialized and highly customizable features, including the duplicated detectors on each color channel for pseudo-autocorrelation, the replaceable emission pinholes for optimal focal point spread function, and the fine-tuning between lasers to maximize the
dual-color overlapping detection volume. The Nikon microscope proved to be able to improve the viability of the cells, thus allowing for more cells collected from each Petri-dish, but yielded similar FCS curve data to those collected in the Gradinaru Lab. Therefore, the live cell dcFCS data collected on both instruments were combined and analyzed without discrimination.

3.2.2.1  In vitro measurements on a coverslip

The in vitro fluorescence correlation measurements were performed following the standard operational procedures for the dcFCS microscope (Chapter 2.1.3). Square microscope glass coverslips (25mm×25mm, thickness #1, VWR) were first rinsed with 95% ethanol then thoroughly cleaned with an argon-plasma cleaner (PDC-32G, Harrick Plasma, USA). The plasma-cleaned surface proved to be free of fluorescent impurities and resistant to non-specific adsorption of most of the samples employed in the current study (GFP- or mCherry- labeled M2 receptors and/or G proteins in detergent solution).

During an in vitro measurement, 30–50μL of the purified sample was added onto the center of the coverslip encircled by a clear reinforcement ring (Avery Products, USA). The concentration of the sample was adjusted such that the fluorescence signal is higher than 10kHz in each detection channel under an excitation laser intensity of 1 – 10kW/cm². A silicone cap was placed on top of the coverslip to prevent evaporation of the sample. Typical data acquisition lasts 0.5 – 2 hours, amounting to a total of 20 – 100 million photons recorded in each channel.

3.2.2.2  Live-cell measurements in a glass-bottom dish

The live cell measurements were performed using glass-bottom Petri-dishes (P35G-1.0-14-C, MatTek, USA). The bottom center of the dish consists of a round piece of thickness No.1 coverslip, allowing the cells to grow on it (Figure 3-2). Subsequent observations can be made directly in the dish, without the need to transfer the cells.

Prior to each measurement, the growth medium in the dish was replaced with colorless imaging medium in which NaHCO₃ was replaced by 25mM HEPES as the pH buffer for
maintaining the optimal pH under regular atmospheric conditions without 5% CO₂ supply.

Figure 3-2. Glass-bottom Petri dish for fluorescence measurement on live cells. The image and diagram were obtained from MatTek website: https://www.mattek.com/products/glass-bottom-dishes.

A Petri dish holder and a horizontal translation stage were installed to facilitate the measurements in live cells in the glass-bottom dishes. Confocal scan imaging was employed to search for cells that showed optimal fluorescence signal intensity. The software-controlled raster scan was carried out with the piezo nano-positioning stage (Mad City Labs Inc.) that is capable of scanning up to a 100 μm by 100 μm area. In practice, there is on average less than one suitable cell in such an area. To measure multiple cells in the same dish, the horizontal translation stage is used to move the dish across a much larger range than that is allowed by the nano-positioning stage. Multiple cells in multiple regions within a single dish can, therefore, be surveyed to improve the data collection efficiency and the statistical reliability of the analysis.

3.2.2.3 FCS fitting program

The analysis of the FCS data files was completed with MATLAB. A single-curve FCS fitting program was initially developed by Greg Gomes in the Gradinaru lab for fitting
single color auto-correlation curves. The program features a graphical user interface and a modular structure that can easily be expanded with additional fitting models (Figure 3-3). A global FCS fitting mode was added to the original program to enable simultaneous fitting of all three correlation curves (green auto-correlation – “AC-Green”, red auto-correlation – “AC-Red”, and cross-correlation – “CC”) output from a dcFCS measurement. The program outputs numerical fitting results in a tabulated .txt file, with the fitted curves and residual plots saved in a .png graph. All FCS data presented in this chapter were analyzed using this custom-written MATLAB program.

Figure 3-3. Screenshot of the custom MATLAB fitting program developed in our lab for fitting FCS curves, showing the dialog window for the choice of fitting models (left), and for fixing or limiting fitting parameters (right).

3.3 Results and Discussion

3.3.1 Single color FCS experiments in detergent micelles

3.3.1.1 GFP multiplexes

To verify the detection efficiency of the instrument and characterize the fluorescence properties of GFP, we performed FCS measurements on purified GFP and GFP-multiplexes. The fitting model including one diffusion component and one triplet decay proved to be sufficient to account for the fluctuations displayed in the experimental FCS curve:


\[
G(\tau) = \frac{1}{\langle N \rangle} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{1}{s^2} \cdot \frac{\tau}{\tau_D}\right)^{-\frac{1}{2}} \left(1 + \frac{f_{wip}}{1 - f_{wip}} \exp\left\{ -\frac{\tau}{\tau_{wip}} \right\} \right)
\]

(Equation 3.1)

where the aspect ratio \( s \) of the 3D Gaussian detection volume is obtained from the calibration measurements performed on fluorescent dyes with known diffusion coefficients (see Chapter 2.1.1, Equation 2.4). The diffusion coefficients \( D \) of the GFP samples are calculated from the fitted diffusion time \( \tau_D \) using Equation 3.2.

\[
D = \frac{w_0^2}{4\tau_D}
\]

(Equation 3.2)

where the size of the beam width \( w_0 \) is obtained from the calibration measurement together with the aspect ratio \( s \) value (Chapter 2.1.1, Equation 2.4). Compared to the diffusion coefficients of standard organic dyes such as Rhodamine 6G (414 \( \mu \text{m}^2/\text{s} \), [31]) and Rhodamine 110 (440 \( \mu \text{m}^2/\text{s} \), [32]), the diffusion coefficient of GFP was found to be much lower, as expected for a much larger molecule. The average value of the diffusion coefficient for the monomeric GFP obtained from five independent measurements was 98\( \pm \)6 \( \mu \text{m}^2/\text{s} \), matching well with literature values (80–100 \( \mu \text{m}^2/\text{s} \) [33, 34]). Furthermore, the diffusion coefficients of GFP multiplexes were determined to be 87\( \pm \)26 \( \mu \text{m}^2/\text{s} \) for the duplex GFP\(_{2x}\), and 50\( \pm \)20 \( \mu \text{m}^2/\text{s} \) for the quadruplex GFP\(_{4x}\). A value of 70–90 \( \mu \text{s} \) for the triplet lifetime was required to fit the FCS curves for all GFP samples, consistent with the same dark-state photophysics affecting the GFP fluorescence.

### 3.3.1.2 Membrane proteins in a detergent buffer

We elected to perform the FCS measurements on purified proteins prior to the measurements in live cells because \textit{in vitro} measurements allow for better-controlled experimental conditions. Because membrane receptors are insoluble in aqueous solution, the \textit{in vitro} measurements require the membrane receptors to be extracted from the production cell line into an amphiphilic detergent solution, through the process known as solubilization. As a result, the receptors reside in micelles formed by the detergent molecules.
Figure 3-4. Schematic illustration of a M₂ receptor solubilized in a micelle. The crystal structure is from the PDB entry 4MQS [35]. The color scale shows the hydrophobicity of the residues of the receptor. The micelle provides an amphiphilic environment that is similar to the native environment of receptors, i.e., the cellular membrane.

Although the micelles do provide an amphiphilic environment similar to the cellular membrane, the conformation of a micelle is noticeably different from the native cellular membrane formed by phospholipids. The shape of the micelles formed by detergent molecules is usually spherical, in contrast to the locally flat lipid bilayer membrane. A spherical micelle encasing the receptors could introduce abnormal tension on the structure of the receptors, while in the meantime affect the interaction between the receptor and other molecules. Most of the structural impacts of detergent micelles on purified membrane proteins are related to the size of the micelle. Therefore, the one-color FCS measurements of receptors in solution in micelles are conducted to retrieve valuable information about the size of the micelles.

The detergents are selected based on previously published studies [26, 27] for the best extraction yield and highest protein stability. In the measurements performed in this project, singly GFP-labeled M₂ receptors (GFP-M₂) are solubilized and purified in digitonin-cholate detergent (0.86% w/v digitonin, 0.17% w/v sodium cholate). On the
other hand, singly GFP-labeled G protein α-subunits (GFP-Gα) are solubilized and purified in sodium cholate detergent (1% w/v sodium cholate).

Autocorrelation curves were measured for GFP-tagged M2 receptor and G protein samples solubilized in detergent micelles (Figure 3-5). Applying the same fitting model (Equation 3.1, 1-component-diffusion-1-triple) on the FCS curves, the diffusion coefficients were measured to be 16±9 μm²/s for the receptor GFP-M2, and 21±9 μm²/s for G protein GFP-Gαβγ from more than five repeated preparations. Both were significantly slower than those measured for GFP multiplexes (as listed above, the diffusion coefficients were 98±6 μm²/s for monomeric GFP; 87±26μm²/s for duplex GFP2X; and 50±20μm²/s for the quadruplex GFP4x).

![Figure 3-5](image)

**Figure 3-5.** Experimental correlation curves for GFP-labeled samples solubilized in detergent micelles, showing the progressively slower diffusion of progressively larger molecular sizes.

The Stokes-Einstein equation was then employed for estimating the hydrodynamic radii of the GFP-labeled samples:

\[
R_H = \frac{k_B T}{6\pi\eta D}
\]  

(Equation 3.3)

where \(k_B\) is the Boltzmann constant, \(T\) is the measurement temperature, \(\eta\) is the viscosity of the solvent, and \(D\) is the diffusion coefficient obtained from FCS fitting. We estimated
that the monomeric GFP has a hydrodynamic radius \((R_H)\) of \(2.2 \pm 1 \text{ nm}\), matching well with values reported from literature (2.82 nm from [36]; 2.3 nm from [37]; 2.40 nm from [38]). The GFP duplex and the quadruplex have \(R_H\) values of \(2.8 \pm 1.4 \text{ nm}\) and \(4.2 \pm 1.6 \text{ nm}\), respectively. This confirms that the calculated radii, within the experimental uncertainty, are clearly increasing with the increasing size of the GFP multiplexes. Moreover, the receptor GFP-M2 and G protein GFP-Gaβγ were estimated to show \(R_H\) values of \(17 \pm 9 \text{ nm}\) and \(12 \pm 5 \text{ nm}\), respectively in their detergent micelles. The estimated radii are larger than those of the GFP multiplexes as expected. However, we found that the large variance in the \(R_H\) values is partly due to the insufficiency of the 1-diffusion-component-1-triplet FCS model (Equation 3.1). The fitting quality of FCS data from the samples dissolved in detergent micelle could be improved by employing a more sophisticated model, from which more physically relevant interpretations of the \(R_H\) values can be made about the receptors and G proteins.

### 3.3.1.3 The 2-component-diffusion FCS model

The detergent micelles are a mixture of different sizes and thus bear different diffusion coefficients. The 1-diffusion-component-1-triplet FCS model lacks the ability to account for such heterogeneity. A natural step to improve the model would be to add in one more diffusion component to help resolve the mixture of diffusion coefficients. The 2-diffusion-component-1-triplet model was introduced as Equations 3.4 and 3.5 below.

\[
G(\tau) = \frac{1}{N} \left[ f_{D1} \left( 1 + \frac{\tau}{\tau_{D1}} \right)^{-1} \left( 1 + \frac{1}{s^2} \cdot \frac{\tau}{\tau_{D1}} \right)^{-\frac{\gamma_2}{2}} + (1 - f_{D1}) \left( 1 + \frac{\tau}{\tau_{D2}} \right)^{-1} \left( 1 + \frac{1}{s^2} \cdot \frac{\tau}{\tau_{D2}} \right)^{-\frac{\gamma_2}{2}} \right] \times \text{Triplet}
\]

with \(\text{Triplet}(\tau) = 1 + \frac{f_{trip}}{1 - f_{trip}} \exp \left( -\frac{\tau}{\tau_{trip}} \right)\)

(Equation 3.4)

The added diffusion component was tested on fitting all the samples containing receptor or G protein in detergent solution. The improvement in fitting quality was confirmed in weighted residual plots and the \(\chi^2\) values. An example is illustrated in Figure 3-6.
Figure 3-6. Comparison of different fitting models for the isolated receptor and G protein in detergent solution. Left: GFP-M2 receptor in digitonin-cholate detergent. Right: GFP-G protein in cholate detergent. The red/green/purple fitted curve and residual were using 1-diffusion-component-1-triplet model, 2-diffusion-component-no-triplet model, and 2-diffusion-component-1-triplet model, respectively. The numerical fitting results are summarized in Table 1 below.

Table 3-1. Summary of the fitting parameters using the three models illustrated in Figure 3.6. The two-component diffusion times, $\tau_{D1}$ and $\tau_{D2}$ from the initial fitting output has been converted to diffusion coefficients $D_1$, $D_2$ according to Equation 3.2.

<table>
<thead>
<tr>
<th>Model</th>
<th>Fitting Results of Receptor</th>
<th>Fitting Results of G protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-diff 1-Tt</td>
<td>2-diff</td>
</tr>
<tr>
<td>$c$ (nM)</td>
<td>4.71</td>
<td>2.84</td>
</tr>
<tr>
<td>$f_{D1}$</td>
<td>-</td>
<td>0.57</td>
</tr>
<tr>
<td>$D_1$ ($\mu m^2/s$)</td>
<td>14.6</td>
<td>131</td>
</tr>
<tr>
<td>$D_2$ ($\mu m^2/s$)</td>
<td>-</td>
<td>11.3</td>
</tr>
<tr>
<td>$f_T$</td>
<td>0.39</td>
<td>-</td>
</tr>
<tr>
<td>$\tau_T$ ($\mu s$)</td>
<td>136</td>
<td>-</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>0.105</td>
<td>0.080</td>
</tr>
</tbody>
</table>
Judging by the residual plots in Figure 3-6 and $\chi^2$ values of the fittings in Table 3-1, it can be noticed that both the triplet term and the second diffusion component term were necessary for satisfactorily fitting the data. Without the second diffusion component, the 1-diffusion-component-1-triplet model was unable to account for the microsecond fluctuations in the correlation curve, yielding unrealistically long triplet lifetime $\tau_T$ (typical triplet relaxation lifetimes are below 100 μs). On the other hand, without the triplet term, the 2-diffusion-component-no-triplet model was still unable to account for the microsecond fluctuations, yielding a fast-diffusing component that is comparable to the free diffusing monomeric GFP, which is unexpected for GFP-labeled proteins in detergent micelles. The 2-component-diffusion-with-1-triplet model showed both better fitting quality (lower $\chi^2$ values and less correlated residual plots) and more physically relevant fitting parameters, and was therefore chosen to fit through all the AC curves collected for receptors and G proteins in detergent micelles.

FCS models with more than two diffusion components have also been applied to studies involving detergent solubilized GPCRs [39-41]. One could argue that the correlated residual plots given by the 2-component-diffusion-with-1-triplet model (Figure 3-6) revealed the insufficiency of the model. The insufficiency was acknowledged because all the tested models used finite number of diffusion components to describe the continuous size distribution of detergent micelles. For the scope of the dcFCS measurement, it is more crucial to faithfully report on the concentration of each fluorescently labeled species than to thoroughly resolve the diffusion coefficients of the components. Therefore, we elected to employ the 2-component-diffusion-with-1-triplet model as the simplest possible form for retrieving relevant physical quantities.

The diffusion coefficients, $D_1$, $D_2$, and hydrodynamic radii, $R_{H1}, R_{H2}$, of each sample were calculated based on the fitted decay times, $\tau_{D1}$ and $\tau_{D2}$, and on the dimensions of the confocal detection volume acquired from the dye calibration measurements according to Equation 3.2. Despite different purification methods used to prepare the samples, the two hydrodynamic radii for isolated M$_2$ receptors and G proteins were consistently found to
cover relatively narrow ranges. For the receptor, \( R_{H1} = 2–3 \text{ nm} \) \((D_1 = 80-100 \ \mu \text{m}^2/\text{s})\) for the fast diffusing component, and \( R_{H2} = 30–42 \text{ nm} \) \((D_2 = 6–9 \ \mu \text{m}^2/\text{s})\) for the slow diffusing component. For the G protein, \( R_{H1} = 2.5–3.5 \text{ nm} \) \((D_1 = 60-80 \ \mu \text{m}^2/\text{s})\) for the fast diffusing component, and \( R_{H2} = 20-30 \text{ nm} \) \((D_2 = 8–11 \ \mu \text{m}^2/\text{s})\) for the slow diffusing component.

The estimated \( R_H \) values for the receptor and G protein samples actually correspond to the physical radii of the detergent micelles enclosing the isolated proteins in our in vitro preparations. The existence of two diffusing species can be attributed to a distribution of different sizes of micelles. Obviously, the radius of the micelle should be larger than the dimensions of the enclosed proteins. The dimensions of the human M2 receptor were estimated by the open-source software HYDROPRO [42] using the published PDB crystal structures 3UON [43], 4MQS, and 4MQT [35]. The hydrodynamic radii for GFP-labeled M2 receptor were computed to be \( 3.2 \pm 0.6 \text{ nm} \). Comparing with the hydrodynamic radii estimated above for the GFP-M2 receptors in digitonin-cholate micelles, it can be concluded that the sizes of the micelles were large enough to accommodate at least one (with \( R_{H1} = 2–3 \text{ nm} \)) and up to several receptors (with \( R_{H2} = 30–42 \text{ nm} \)). The distribution range of the hydrodynamic radii matched well with the bulk dynamic light scattering spectroscopy results measured with the digitonin-cholate detergent (Figure 3-7). It was therefore concluded that the size of the digitonin-cholate micelles seemed large enough for hosting the receptors and preserving their physiological conformation, as suggested by previous studies [44-46].

![Figure 3-7](image_url)  
**Figure 3-7.** Dynamic Light Scattering spectroscopy result on the digitonin-cholate detergent. (Equipment model: Zetasizer Nano ZS, Malvern Instruments Ltd.) Two major hydrodynamic
radii populations were detected at $R_{H1} = 5.0 \pm 1.6$ nm (with 62% of total intensity) and $R_{H2} = 125 \pm 30$ nm (with 25% of total intensity).

Similarly, the dimensions of the G protein heterotrimers were estimated by HYDROPRO [42] using PDB crystal structures 5KDL [47], 4N0E [48], and 3FFB [49]. The hydrodynamic radius for GFP-labeled G protein heterotrimer was computed to be $4.0 \pm 0.8$ nm. Comparing with the hydrodynamic radii estimated above for the G proteins in cholate micelles, it was concluded that there are micelles large enough to contain at least one (with $R_{H1} = 2.5–3.5$ nm), and up to several G proteins (with $R_{H2} = 20–30$ nm).

3.3.2 Dual color FCS experiments in detergent micelles

3.3.2.1 Fitting of the cross-correlation curve

When fitting the whole suite of three correlation curves measured in a dcFCS measurement, the AC curves are well fitted with the 2-component-diffusion-with-1-triplet model as discussed above. However, the triplet term was not needed when fitting the CC curves; this is physically reasonable because the dark-state photophysics of one fluorophore is fundamentally uncorrelated with the dark-state photophysics of the other fluorophore. Therefore, the 2-component-diffusion-with-no-triplet model was chosen to fit the cross-correlation curves.

\[
G(\tau) = \frac{1}{\langle N \rangle} \left[ f_{D1} \left(1+\frac{\tau}{\tau_{D1}}\right)^{-1} \left(1+\frac{1}{s^2} \cdot \frac{\tau}{\tau_{D1}}\right)^{-\frac{\tau}{\tau_{D1}}} + (1-f_{D1}) \left(1+\frac{\tau}{\tau_{D2}}\right)^{-1} \left(1+\frac{1}{s^2} \cdot \frac{\tau}{\tau_{D2}}\right)^{-\frac{\tau}{\tau_{D2}}} \right]
\]

(Equation 3.5)

The three correlation curves from a dcFCS measurement – AC-green, AC-red, and CC – are fitted globally with our custom-written MATLAB fitting program. The lifetimes of the two diffusion components, $\tau_{D1}$ and $\tau_{D2}$, were shared among the three curves, whereas the fractional contributions, $f_{D1}$, were allowed to vary independently.

On a technical note, we found that due to the complexity of the parametric space, fitting of dcFCS curves is generally sensitive to initialization values and fitting parameter
constraints. It is therefore recommended to use highly customizable fitting programs to explore different fitting models and adapt to the constraints posed by the system.

Using the amplitudes of the correlation curves, \( G_g(0) \), \( G_r(0) \), and \( G_x(0) \), the fraction of co-diffusion (\( fcd \)) of each of the two fluorescently labeled molecules can be calculated according to the methods mentioned in Chapter 2.1.3:

\[
\begin{align*}
  fcd_g &= \frac{c_x}{c_g} = \frac{G_x(0)}{G_g(0)} \cdot \text{OVCF}_g, \\
  fcd_r &= \frac{c_x}{c_r} = \frac{G_x(0)}{G_r(0)} \cdot \text{OVCF}_r.
\end{align*}
\]

(Equation 3.6)

where \( \text{OVCF}_g \equiv \left( \frac{V_g}{V_{eff,x}} \right) \) ; \( \text{OVCF}_r \equiv \left( \frac{V_r}{V_{eff,x}} \right) \).

The overlap volume correction factors (OVCF) for the green (GFP) and red (mCh) detection channels for each batch of \textit{in vitro} measurements were measured with dually labeled DNA controls.

### 3.3.2.2 \textit{In vitro} two-color control samples

As the dcFCS setup was regularly checked and re-aligned, control experiments were performed to characterize the upper and lower limit of the cross-correlation amplitudes. The samples included the dually labeled dsDNA as a positive control, and an equimolar solution mixture of the two dyes used for labelling as a negative control. The dsDNA tagged with two dyes (6-FAM and Texas615) typically gave cross-to-auto-correlation ratios of \( fcd_g = 0.70-0.80 \) (green channel) and \( fcd_r = 0.50-0.65 \) (red channel). These values are indicative of the spatial overlap between the two spectral detection volumes. Since dcFCS analysis for the dually labeled dsDNA sample should yield \( fcd \) values of nearly 100%, the experimental cross-to-auto-correlation ratios obtained for this dsDNA sample were used to calculate OVCF according to Chapter 2.1.4.6. The \( fcd \) in the subsequent dcFCS measurements performed on M2 receptors and G proteins were then calculated using the procedure outlined in Chapter 2.1.2. In practice, the alignment of the dcFCS microscope was stable for at least one week. Therefore, all the measurements finished in the same week shared the same OVCF values.
3.3.2.3 The coupling of Ga and Gβγ subunits

We measured the co-diffusion of Ga subunit and Gβγ subunits by co-expressing mCh-Ga with GFP-Gβγ followed by extraction and purification in cholate detergent. On the dcFCS microscope, the two subunits showed significant fractions of cross-correlation. Averaged from six independent measurements, the fcd were found to be 48.6 ± 10.6% of mCh-Ga and 70.2 ± 13.4% of GFP-Gβγ after correcting for the OVCF. The amplitude of the CC curve (Figure 3-8) indicates that a significant fraction of the fluorescently tagged G proteins was successfully assembled as heterotrimeric G proteins after the extraction and purification process. This was a favorable result because physiologically any relevant FCS measurement on the G proteins would require that the fluorescent labels do not seriously impede the function of G proteins.

Figure 3-8. dcFCS measurement of the coupling between mCh-Ga and GFP-Gβγ subunits solubilized in detergent micelles, showing the correlation curves after the correction for OVCF. High fractions of co-diffusion (ratios of CC to AC) indicate that mCh-Ga and GFP-Gβγ successfully formed coupled heterotrimeric Gaβγ complexes.

3.3.2.4 Oligomers of receptors in digitonin-cholate micelles

To observe the possible oligomerization of M2 receptors, we co-expressed GFP- and mCherry-labeled receptors. The polyhistidine His6-tag and the FLAG-tag were
incorporated into the GFP and mCherry sequences, respectively. After extraction from the Sf9 cells and purification through both the Ni-NTA column and the anti-FLAG column, 80.5±17.5% of M2-GFP and 38.5±19.1% of M2-mCh were found to co-diffuse with each other based on three independent dcFCS measurements. The existence of coupled diffusion of differently labeled M2 receptors indicates that the M2 receptors form oligomers that are at least dimeric in size.

Figure 3-9. dcFCS measurement of the coupling between co-purified GFP-M2 and mCh-M2 receptors, showing the correlation curves after the correction for OVCF. High fractions of co-diffusion indicate that there were GFP-labeled receptors coupled with mCh-labeled receptors, forming oligomers with a size of at least two.

The existence of M2 oligomers was anticipated based on published pharmacological assays such as Co-ImmunoPrecipitation (Co-IP) and radioligand binding [50, 51]. The size of oligomers cannot be accurately estimated with the pharmacological measurements or dcFCS measurement, and will be addressed in Chapter 4 using single-molecule photobleaching stoichiometry.

3.3.2.5 Oligomers of G proteins in cholate micelles

To observe the possible oligomerization of G protein, we co-expressed His6-tagged GFP-Gα subunits and His6-tagged mCh-Gα subunit, in the presence of unlabeled wild-type
Gβγ subunits. After extraction from the sf9 cells and purified through the Ni-NTA column, 53.5±20.3% of GFP-Gα protein and 53.9±15.1% of mCh-Gα protein were found to co-diffuse with each other based on three independent dcFCS measurements, indicating the presence of oligomeric G proteins in the purified samples.

Figure 3-10. dcFCS measurement of the coupling between co-purified GFP-Gα and mCh-Gα subunits in cholate detergent, showing the correlation curves after the correction for OVCF. High fractions of co-diffusion indicate that there were GFP-labeled Gα subunits coupled with mCh-labeled Gα subunits, forming oligomers with a size of at least two.

In recent years, as much effort was devoted to investigating the oligomeric status of various GPCRs, the oligomeric status of G proteins was mostly disregarded with the exception of a few early in vitro studies [52, 53]. Considering the fact that the M2 receptors couple with G_{i1} proteins in a 1:1 ratio [50], our results highlighted the possibility that the M2 signaling complex are composed of oligomeric receptors and oligomeric G proteins.

3.3.2.6 Coupling between M2 receptors and G proteins measured in isolated complexes

The receptor-G protein coupling experiments need carefully designed constructs. As part of the GPCR signaling pathways, GTP binding to the activated G protein will lead to the
rapid detachment of G protein from the receptor. Consequently, the agonist-induced coupling state between the receptor and G protein could be very short-lived in live cells due to access to endogenous GTP (with a time scale less than 1 second, considering the signaling efficiency of the receptors [9, 10]). Due to the transient nature of this coupling, it may be difficult to isolate a significant amount of RG complexes. In addition, the acquisition for a correlation curve is typically longer than 10 seconds per segment (Chapter 2.1.1), which is significantly longer than the receptor-G protein coupling time. Therefore, our initial live cell measurements of the coupling between M2 receptor and G proteins showed close to zero cross-correlations regardless of the presence of agonist (data not shown).

To enable the dcFCS technique for receptor-G protein interaction studies, we employed a modified G protein with the mutation N270D in the Gα subunit. This mutant has been reported to have a normal binding affinity to the receptor, but impaired dissociation from the activated receptor [12, 54], thus stabilizing the coupled receptor-G protein complex for the duration of the measurement.

The fractions of co-diffusing species for the basal state sample, “RGHisFlag”, was 4.0% of M2-GFP and 10.7% of mCh-Gαi. The complex extracted after adding agonist (10μM carbachol), “RGHisFlag+car”, showed a significant increase in fractions of co-diffusion: 51.4% of M2-GFP and 13.8% of mCh-Gαi. Furthermore, the complex extracted after adding antagonist (10μM “NMS” (N-methylscopolamine)), “RGHisFlag+nms” showed basal-state levels of CC amplitude, corresponding to 3.9% of M2-GFP and 13.0% of mCh-Gαi co-diffusing with each other.

The response of receptor-G protein coupling upon addition of different ligands suggests that a majority of the M2 receptors and the Gi1 proteins are not pre-coupled in the absence of agonist, thus favoring the scenario in which G proteins are transiently recruited and coupled to the receptor upon receptor activation.
Figure 3-11. dcFCS measurements of the coupling between the M₂-GFP receptor and mCh-Gα subunit in samples isolated from untreated cells (A), cells treated with the agonist carbachol (B) or with the antagonist NMS (C).

3.3.2.7 Co-diffusion is not an artifact of detergent purification

With all the above-mentioned measurements performed in detergent, a question arises whether the observed co-diffusion behavior is an artifact caused by enclosure in detergent micelles. To address this, we computed the correlation between the fraction of co-diffusion ($f_{cd}$) and the diffusion coefficient $D$, considering both “fast” and “slow” components simultaneously.

The concentration of detergent micelles was much higher than concentration of purified molecules in all the *in vitro* measurements. The composition of receptor detergent micelles (0.86%-0.17% digitonin-cholate) corresponded to an expected detergent micelle
concentration of 0.12 mM. The composition of G protein detergent micelles (1% sodium cholate) corresponded to an expected detergent micelle concentration of 1.5 mM. Both concentrations were much larger than the measured concentrations of receptor or G protein samples, which were typically 0.1~50 nM. The excessive amount of micelles ensured that the interactions between receptors and G proteins were not due to insufficient micelle enclosure.

The influence of the size of the micelle on the co-diffusion of solubilized molecules can be estimated with FCS. The 2-component-diffusion FCS model directly outputs the diffusion coefficients of the two diffusing components that are shared among all FCS curves (D₁ and D₂), the fractions of photons emitted from the fast diffusion components (f_{D₁,g}, f_{D₁,r}, f_{D₁,x}), and the fractions from the slow diffusing components (f_{D₂,g}, f_{D₂,r}, f_{D₂,x}). The fractions of co-diffusion for the fast green species (f_{cd_{D₁}}), the slow green species (f_{cd_{D₂}}), the fast red species (f_{cd_{r,D₁}}), and the slow red species (f_{cd_{r,D₂}}) are calculated individually from the amplitudes of AC and CC curves according to Equation 3.7.

\[
\begin{align*}
    f_{cd_{D₁}} &= \frac{f_{D₁,x}}{f_{D₁,g}} \cdot \frac{c_x}{c_g} = \frac{f_{D₁,x}}{f_{D₁,g}} \cdot \frac{G_x(0)}{G_r(0)} \cdot \text{OVCF}_g \\
    f_{cd_{D₂}} &= \frac{(1 - f_{D₁,g}) \cdot c_x}{(1 - f_{D₁,g}) \cdot c_g} = \frac{(1 - f_{D₁,g}) \cdot G_x(0)}{(1 - f_{D₁,g}) \cdot G_r(0)} \cdot \text{OVCF}_g \\
    f_{cd_{r,D₁}} &= \frac{f_{D₁,r}}{f_{D₁,g}} \cdot \frac{c_r}{c_g} = \frac{f_{D₁,r}}{f_{D₁,g}} \cdot \frac{G_x(0)}{G_r(0)} \cdot \text{OVCF}_r \\
    f_{cd_{r,D₂}} &= \frac{(1 - f_{D₁,g}) \cdot c_x}{(1 - f_{D₁,g}) \cdot c_g} = \frac{(1 - f_{D₁,g}) \cdot G_x(0)}{(1 - f_{D₁,g}) \cdot G_r(0)} \cdot \text{OVCF}_r
\end{align*}
\]

(Equation 3.7)

For all the in vitro dcFCS results, the calculated fcd values are all grouped together and plotted against their corresponding D values in Figure 3-12. Linear regression was performed on the GFP and the mCh data to detect any dependence between fcd and D. For the GFP-labeled samples, the linear regression yielded a R² value (i.e. the value of the Pearson correlation coefficient) of 0.077 and a slope value of -0.10±0.07, indicating very weak correlation between fcd_{g} and D_{g}, and no evidence against the null hypothesis that the linear dependence between fcd_{g} and D_{g} is zero (p = 0.21). For mCh-labeled samples, the linear regression yielded an R² value of 0.20 and a slope value of -
0.19±0.08, indicating weak correlation between $f_{cd}$ and $D$, and weak evidence against the null hypothesis that the linear dependence between $f_{cd}$ and $D$ is zero ($p = 0.08$). The size of the micelle proved to be not influential on the measured fractions of co-diffusion.

**Figure 3-12.** The plot of the fitted $f_{cd}$ vs. $D$ for all the *in vitro* experiments. The $f_{cd}$ for the fast diffusing component and the $f_{cd}$ of the slow diffusing component were calculated separately using Equation 3.7, and then grouped together. The dotted lines are the linear regression trendlines for each fluorophore.

### 3.3.3 Preparation and control experiments in live cells

Although elegant and insightful, the *in vitro* measurements suffer from a critical drawback: over-simplification. The well-controlled environmental conditions in a purified sample can impact the “native state” of the molecules of interest. In particular, the detergent micelle is clearly distinct from the cellular membrane both in topology and in composition. Consequently, purified membrane proteins could exhibit different intra- and inter-molecular interactions. To study the M2 receptors in a more physiologically relevant environment, we moved on to perform in-cell FCS experiments.
3.3.3.1 Confocal scan imaging of the cells

Fluorescently labeled M₂ receptors and G proteins were expressed in the CHO cells through transient transfection. The carrier cells were grown on the coverslip at the bottom of glass-bottom Petri-dishes (Figure 3-2). The expression level of the transfected proteins was monitored regularly using a bench-top inverted UV fluorescence microscope (Amscope XYL403, Biocotek Ltd.). After verifying for successful transfection, the dish with CHO cells was mounted on our setup for live cell dual-color fluorescence correlation measurements. Due to the intrinsic variance of individual cells, scanning confocal imaging had to be performed prior to dcFCS measurements to locate and screen for cells with the desired properties. Cells with saturating or insufficient fluorescence brightness, or showing signs of apoptosis (such as shrinkage in size or membrane fragmentation [55]) were not examined with dcFCS.
**Figure 3-13.** Confocal scan images of CHO cells expressing: (A) Cytosolic GFP, (B) membrane-anchored MP-GFP, (C) GFP-Gα subunit, and (D) GFP-M2. All images have the area size of 20μm × 20μm. Note that membrane bound proteins localize near the cell membrane, as expected.

Confocal scanning images of cells expressing GFP-labeled proteins are shown in Figure 3-13. Cytosolic GFPs showed a uniform distribution of fluorescence within the whole cell. MP-GFP (GFP with the Myristoylation-Palmitoylation membrane anchoring modification) and M2-GFP (M2 receptor labeled with GFP on the extracellular end) had most of the fluorescence localized on the membrane, indicating successful membrane targeting of these proteins. The GFP-Gα subunit exhibited a distribution of fluorescence that is both on the membrane and in the cytoplasm, however with significantly higher density near the cell membrane.

### 3.3.3.2 FCS artifacts of cell motion

In-cell measurements were preferred over in-solution measurements for the ability to observe the bio-macromolecules in their native environment and for the avoidance of delicate/complex purification steps. However, these advantages come at the cost of more complicated and uncontrolled measurement conditions and a higher propensity for experimental artifacts. FCS measurements in a confocal fluorescence microscope avoid many artifacts by employing bandpass emission filters and spatial-filtering pinholes. However, other sources of unwanted fluctuations including membrane flexibility, overall cell motility, spot-to-spot and cell-to-cell variability remain unaccounted.

Because FCS focuses on uncovering the information related to temporal variations of the detected signal, this technique is particularly sensitive to noise and unwanted fluctuations. A cell, as a dynamic system, is a rich source of spatial-temporal fluctuations that can complicate the diffusion analysis of an FCS curve. One of the most influential sources of FCS artifacts is membrane motion. The large-scale motion of the cellular membrane can create a highly unstable fluorescence intensity trace, and a “dive-like” feature in the FCS curve around the lag time of τ = 1s–100s (see an example in Figure 3-14 below).
Figure 3-14. Illustration of FCS artifacts caused by cell motion. The dcFCS measurement on co-expressed GFP-M₃ and mCh-Gα failed due to excessive fluctuation in fluorescence intensity (Panel A). The resultant FCS curves showed a “plateau-dive” shape that cannot be explained with diffusion-based FCS models (Panel B).

Fortunately, this membrane motion occurs on a longer time scale than the typical translational diffusion of proteins in the cell membrane. Therefore, the artifact can often be countered by subtracting the whole correlation curve by the correlation amplitude value at 1s lag time, i.e. ignoring all fluctuations that are slower than 1s. However, this simple subtraction method cannot remove all of the artifacts caused by cellular activity. Other sources of fluctuations include interactions with endogenous proteins, active transportation of the expressed proteins by molecular motors, and cellular autofluorescence. The consequence is that measurements on multiple cells are needed to draw robust conclusions regarding fcd in dcFCS measurements. A certain behavior observed on one single cell, even though prominent, could be the result of cell-to-cell diversity. Repeated measurements on multiple cells can help rule out such possibilities.
3.3.3.3 FCS measurements at bottom vs side vs top membrane

![Confocal scan images of a cell expressing M2-GFP](image)

**Figure 3-15.** Confocal scan images of a cell expressing M2-GFP, sliced at z-12 (near the top membrane), z-8, z-4 (in the middle of the cell), and z-0 (near the bottom membrane). All images have the same area size of 30μm × 30μm. Fluorescence intensity scale unit: kHz.

For the receptor and G protein experiments, we found that the chance of observing stable FCS curves depends on the position of the confocal measuring spot in the cell. Figure 3-15 shows a series of confocal scan images of a CHO cell at different depths above the coverslip surface. It can be seen that the apparent distribution of fluorescence varies at different sectioning planes.

FCS measurements were performed on the top membrane, the side membrane, and the bottom membrane. When measurements were successfully completed without cell motion artifacts, there is no substantial difference in terms of diffusion component and fluorophore photophysics between the FCS curves collected at different locations on the cell. However, in general, the top membrane was the least prone to cell (membrane)
motion artifacts, but suffered from the most amount of noise in the correlation amplitudes, possibly due to a large amount of uncorrelated background fluorescence from outside of the membrane (intracellular or extracellular autofluorescence). Secondly, while the side membrane was most favorable in terms of excluding out-of-membrane signals, it was the most highly susceptible to cell motion artifacts. Finally, FCS measurements at the bottom cell membrane were least susceptible to cell motion effects and were chosen for the majority (~70%) of the experiments described in the current research. FCS measurements on the bottom membrane were typically performed at bright regions with low excitation intensity (<100W/cm²) to prevent photobleaching; regions with low fluorescence were avoided for reducing uncorrelated autofluorescence noise; regions with many discernable mobile particles were avoided because of the propensity to correlation artifacts caused by the motion of the particles.

3.3.3.4 Obtaining weighting information for live cell data

Fitting FCS curves requires the standard deviation (S.D.) at each data point as the weighting function. Traditionally, the S.D. is calculated from multiple measurements repeated at identical experimental conditions. With the difficulty to maintain the cell at a stable state for an extended period of time, the FCS measurements in cells rarely allowed for more than 300 seconds of continuous collection time. In addition, different cells usually displayed different behaviors. Therefore, it is challenging to acquire the S.D. information from repeated measurements over different cells. In other words, fitting of the live cell FCS curves does not have access to the same type of weighting functions that were available for *in vitro* measurements via averaging over repeated data. According to Wohland *et. al.* [56], the theoretical S.D. can be used instead of the empirical S.D. However, the theoretical S.D. requires the internal register readings of the total number of photons acquired in each lag time channel of the correlator and is inaccessible in our setup.

We devised an alternative weighting method by taking the “sliding window” S.D. along the correlation curve, *i.e.* the standard deviation at each lag time $\tau$ is calculated by taking the standard deviation of (typically five) nearest data points (discarding two data points both at the beginning and at the end of the correlation curve).
\[ SD(\tau_i) = SD(G(\tau_{i-2}), G(\tau_{i-1}), G(\tau_i), G(\tau_{i+1}), G(\tau_{i+2})) \]  

(Equation 3.8)

This alternative method was verified by comparing the “sliding window S.D.” with the empirical S.D. as shown in Figure 3-16. Figure 3-16 (A) shows the case where the noise (for this sample, the source of noise is the cellular activity-related fluctuations) was significant and comparable to the fluorescence fluctuations caused by diffusing fluorophores. In this case, the “sliding window S.D.” showed a trend very similar to the empirical S.D. The “sliding window S.D.” therefore should enable a relative weighting of the FCS curves whenever the FCS curve is noisy but the empirical S.D. is not available. Conversely, Figure 3-16 (B) shows the case where there is no excessive noise (a pure fluorescent dye solution). In this case, the “sliding window S.D.” could fail for the fact that the transition region of the sigmoidal curve will be translated into high S.D. values, leading to errors in the estimation of the diffusion time. Another drawback of the “sliding window S.D.” is that the reported \( \chi^2 \) values (i.e. wrSS, “weighted residual sum of squares“) from the fitting may not reflect the true fitting quality. Consequently, the choice of fitting models has to be made based on the residual plots and the practical interpretation of fitting parameters in addition to the weighted \( \chi^2 \) values.

Figure 3-16. Comparison of “sliding window S.D.” (red curves) vs. “empirical S.D.” (black curves). The standard deviation curves were calculated for (A) FCS curve of GFP-G\( \alpha \) in live cells, where the noise is significant; and for (B) FCS curve of a calibration dye molecule Rhodamine 110 in aqueous PBS buffer, where the noise is much smaller compared to the amplitude. The corresponding FCS curves are shown in the insets. Note that the sliding window
S.D. incorrectly interpreted the “transition region” of the FCS curve with low noise (case B) as a higher standard deviation.

3.3.4 Single color FCS measurements in live cells

3.3.4.1 The 1-component-anomalous-diffusion fitting model

With the prior knowledge that 2-component-normal-diffusion model fitted best for the in vitro 1-color FCS curves (Section 3.3.1.3), we tried fitting the live cell FCS data from GFP-\( \text{G}_\alpha \) with four different (two-dimensional diffusion) models: 1-component-normal-diffusion, 2-component-normal-diffusion, 1-component-anomalous-diffusion, and 2-component-anomalous-diffusion.

The 1-component-normal-diffusion model resulted in large \( \chi^2 \) values and significantly correlated residuals, indicating the existence of additional decay processes that were not accounted for. The other three more complex models yielded smaller \( \chi^2 \) values. Both of the 2-component-normal-diffusion model and the 2-component-anomalous-diffusion model were discarded for frequently yielding degenerated diffusion components that matched those retrieved from the 1-component-diffusion model. We eventually determined the 1-component-anomalous-diffusion model (Equation 3.9) as the best performing model among all, with which we fitted all live cell FCS curves. The anomalous factor \( \alpha \) retrieved from all of the fluorescently labeled proteins in live cells remained consistently within the range of 0.5–0.8, indicating an obstructed diffusion behavior that could be successfully explained by the anomalous model.

\[
G(\tau) = \frac{1}{\langle N \rangle} \left[ 1 + \left( \frac{\tau}{\tau_D} \right)^\alpha \right]^{\langle \chi \rangle^2} \left( 1 + \frac{1}{\langle s^2 \rangle} \left( \frac{\tau}{\tau_D} \right)^\alpha \right)^{-\langle \chi \rangle^2} \left( 1 + \frac{f_{\text{trip}}}{1 - f_{\text{trip}}} \exp \left\{ -\frac{\tau}{\tau_{\text{trip}}} \right\} \right) \quad (\text{Equation 3.9})
\]

Note that the abovementioned fitting model for live-cell measurements of the receptors and G proteins is a 2D diffusion model, considering the fact that receptors and G proteins are mostly diffusing on or near the cellular membrane. The \( s \) parameter (the aspect ratio of the 3D Gaussian excitation/detection volume) was fixed to 1 for measurements on the bottom or top cellular membrane (\textit{i.e.} diffusion in the \( x \)-\( y \) plane), or fixed to the actual
aspect ratio (as determined by calibration dyes) for measurements on the side cellular membrane (i.e. diffusion in the x-z plane).

Furthermore, we tested the inclusion of triplet components in the fitting model. Although triplets improved the $\chi^2$ value and reduced the correlated structure of the residual plot, the triplet fitting parameters (lifetime and population fraction) tended to remain very close to the initialization values. Considering the relatively large noise of the correlation curves in the microsecond time range, it is possible that the fitting is badly convergent for fitting triplet decays. Therefore, we recommend caution against employing triplets for fitting in-cell FCS data or over-interpreting the triplet fitting parameters obtained from these data.

**Figure 3-17.** Comparison of FCS curves measured for Rhodamine 110 (“R110”), GFP in solution (“GFPsol”), GFP in the cytoplasm (“GFPcyto”), membrane-anchored GFP (“MP-GFP”), and GFP-M$_2$ in live CHO cells. The data show significantly slower diffusion for proteins in the cytoplasm than those in the aqueous solution, and even slower for those in the cell membrane.

The fitted diffusion coefficients for the data shown in Figure 3-17 were 97.6$\mu$m$^2$/s for GFP in solution (“GFPsol”), 14.2$\mu$m$^2$/s for GFP in the cytoplasm (“GFPcyto”), 0.121$\mu$m$^2$/s for membrane-anchored GFP (“MP-GFP”), and 0.132$\mu$m$^2$/s for the GFP-tagged receptor (“GFP-M$_2$”). These changes in the values are in agreement with previously published results for the diffusion of GFP-tagged proteins in aqueous solution [34], in the cell cytoplasm [33, 57] and in the cellular membrane [58, 59]. Samples in
solution ("GFPsol") and in the cytoplasm ("GFPcyto") gave rise to FCS curves described by a single diffusing species (Equation 3.1). On the other hand, the samples at the cell membrane, “MP-GFP” and “GFP-M₂”, exhibit less steep correlation decay curves. They can be described by anomalous diffusion (Equation 3.9) which is most likely caused by crowding [60-62].

3.3.5 Dual color FCS measurements in live cells

Dual-color FCS measurements on live CHO cells were performed using concomitant blue (473 nm) and green (532 nm) laser to excite GFP- and mCherry-labeled proteins, respectively. In the majority of samples, we transfected a larger amount of plasmids encoding the mCherry-labeled species than those encoding the GFP-labeled species, to counteract the excitation/different detection efficiency for the two fluorophores on our setup. A ratio of mCherry plasmids to GFP plasmids of 2:1 to 2.5:1 yielded a large fraction of cells with similar GFP and mCherry fluorescence signal intensities. Only cells with mCherry fluorescence intensity above 50% of the GFP intensity were chosen for dcFCS data acquisition and analysis. This ensured minimal artifacts due to spectral bleed-through from the green to the red detection channel while maintaining the two autocorrelation amplitudes at similar levels. The three resulting dcFCS curves for each experiment were fitted globally to the 1-component anomalous-diffusion model (Equation 3.9), with anomalous factor α shared among all the curves.

3.3.5.1 Dual color live-cell control measurements

To test the performance of the dcFCS in live cells, we measured cross-correlation between two fully coupled molecules, the Gα and Gβ subunits of the Gi protein, and between two unrelated molecules, the MP-GFP and the mCh-Gα. The three subunits of Gi protein, Gα₁, Gβ₁, and Gγ₂ were transfected into live CHO cells simultaneously. With mCherry attached to the Gα subunit and GFP to the Gβ subunit, we were able to test the performance of the dcFCS technique in live cells by measuring the cross-correlation between the GFP and mCherry fluorescence signals. Without an external stimulus, the three subunits of the heterotrimeric G protein are expected to always couple with each other, whereas the MP-GFP and mCh-Gα are expected to not couple with each other.
On the cellular scale, the Gα and Gβ seem to be co-localized, as seen from the confocal fluorescence images of the CHO cells co-expressing mCh-Gα, GFP-Gβ, and Gγ (Figure 3-18, A-B). Significant fractions of coupled mCh-Gα (75±32%) and GFP-Gβ (87±25%) were observed based on the cross-correlation amplitudes (Figure 3-18, C). However, the \( f_{cd} \) (fraction of co-diffusion) varied from cell to cell, and were always lower than 100% after correction for the overlap volume using the OVCFs obtained from double-labeled DNA. This was attributed partly to the discrepancy between 2D diffusion (for membrane proteins) and 3D diffusion (for the DNA). The OVCFs calculated for 3D Gaussian detection volumes might not be directly applied to 2D cases. In addition, one cannot rule out the possibility that parts of the observed subunits were not fully folded, thus unable to couple with the other subunits. Although the concentration of endogenous G proteins was negligible given the levels of transfected G proteins (Figure 3-1), it is still possible that endogenous unlabeled G protein subunits reduced the fraction of dually labeled heterotrimers by coupling with the labeled subunits.

Due to the lack of a reliable dually-labeled control sample in live cells, the above-measured fractions of coupled Gα and Gβ subunits were treated as the expected \( f_{cd} \) for a sample that is actually fully coupled. Moreover, no OVCF was applied for subsequent dcFCS measurements in live cells. The consequence is that the calculated \( f_{cd} \) values no longer represent the true fractions of co-diffusion. Instead, the calculated \( f_{cd} \) values from different experimental conditions were compared relatively with each other.
Figure 3-18. Confocal fluorescence images and dcFCS measurements of two control samples: (A–C) the positive control, mCh-Ga, GFP-Gβ with Gγ; and (D–F) the negative control, MP-GFP and mCh-Ga. Both samples appear co-localized on the confocal images, but the positive control (C) showed significant fractions of co-diffusion (69% of GFP-Gβ and 73% of mCh-Ga were co-diffusing); whereas the negative control (F) showed nearly zero fractions of co-diffusion (8.9% of MP-GFP and 6.6% of mCh-Ga were co-diffusing).

We further measured a negative control sample consisting of MP-GFP and mCh-Ga co-expressed in the CHO cells. No significant cross-correlation was observed between the
GFP and mCherry signals (Figure 3-18, F. *fcd* less than 10% for both GFP and mCh). However, judging by the confocal fluorescence images (Figure 3-18, D-E), the MP-GFP and mCh-Gα signal appeared to co-localize with each other. This indicated that the fraction of co-diffusion cannot be confirmed by the microscopy images alone, and correlation spectroscopy is necessary for studying the coupling effect. The negligible *fcd* between MP-GFP and mCh-Gα confirmed that the cross-correlation measured for G protein heterotrimers originated from protein complexes containing both fluorophores rather than from a spectral bleed-through effect. In addition, this also confirms that the coupling effects observed between GFP- and mCherry-labeled molecules in live cells were not an artifact induced by interactions between the GFP and mCherry proteins.

Lastly, we examined the coupling between Gα and Gβ subunits in the presence of the M2 receptor with and without agonist ligand. Wild type M2 receptors were co-expressed with mCh-Gα, GFP-Gβ and Gγ subunits. When an agonist (10 mM carbachol) was added to the cells, the cross-correlation between GFP and mCherry signals decreased considerably to 59±19% of GFP-Gβ and 26±7% of mCh-Gα, corresponding to an induced separation of Gα and Gβ subunits. This decoupling of the G protein subunits upon receptor activation is consistent with the widely accepted signaling pathway of the G proteins [11, 13, 63]. The dcFCS results of the control measurements on double-labeled heterotrimeric G proteins are summarized in Table 3-2.

Table 3-2. Summary of the dcFCS analysis of the coupling interactions between G protein subunits in live cells.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sample composition</th>
<th># of cells</th>
<th><em>fcd</em> green</th>
<th><em>fcd</em> red</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gαβγ</td>
<td>GαiND-mCh + Gβ-GFP + Gγ</td>
<td>14</td>
<td>0.87 ± 0.25</td>
<td>0.75 ± 0.32</td>
</tr>
<tr>
<td>(R)Gαβγ</td>
<td>M2 + GαiND-mCh + Gβ-GFP + Gγ</td>
<td>8</td>
<td>0.8 ± 0.26</td>
<td>0.61 ± 0.24</td>
</tr>
<tr>
<td>(R)Gαβγ+ag</td>
<td>M2 + GαiND-mCh + Gβ-GFP + Gγ + carbachol</td>
<td>5</td>
<td>0.59 ± 0.19</td>
<td>0.26 ± 0.07</td>
</tr>
</tbody>
</table>

3.3.5.2 Evidence for RR oligomerization in live cells

We co-expressed M2-GFP and M2-mCh and used the dcFCS technique to study the oligomerization status of M2 receptors in live CHO cells. Despite the signal being influenced by cellular motion artifacts, the cross-correlation curves consistently showed
significant non-zero fractions of co-diffusing receptors (Figure 3-19). This indicates the presence of M$_2$ oligomers in live cells and is consistent with the dcFCS results obtained for the isolated samples (Figure 3-9). Among a total of 10 different cells measured, there was an average fraction of 42±23% M$_2$-GFP and 35±18% of M$_2$-mCh that were coupled to each other (Figure 3-19). The fractions of co-diffusion/coupling did not appear to change upon the introduction of agonist (10mM carbachol), as 45±20% M$_2$-GFP and 32±19% of M$_2$-mCh remained coupled after incubation with the agonist. The significant cross-correlation amplitude is an unambiguous indicator of the existence of M$_2$ oligomers. Quantifying the exact size(s) of the oligomer(s) is beyond the scope and the sensitivity of the dcFCS technique, and will be addressed using the single-molecule photobleaching technique in Chapter 4.

![Graph](image.png)

**Figure 3-19.** dcFCS measurement of GFP-M$_2$ and mCh-M$_2$ in live cells, showing significant fractions of co-diffusion (48% of GFP-M$_2$, 33% of mCh-M$_2$).

The dcFCS results of the co-expressed GFP-M$_2$ and mCh-M$_2$ receptors are summarized in Table 3-3. The significant fractions of co-diffusion indicated the existence of oligomeric M$_2$ receptors with sizes of at least 2. The evidence of oligomeric M$_2$ receptors in live cells support the previously published literature with multiple techniques including single-color FCS measurements, molecular brightness measurements, and quantitative FRET measurements, etc. [64, 65]
Table 3-3. Summary of the dcFCS analysis of coupling between GFP-M₂ and mCh-M₂ receptors in live cells.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sample composition</th>
<th># of cells</th>
<th>fcd green</th>
<th>fcd red</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR</td>
<td>M₂-GFP + M₂-mCh</td>
<td>10</td>
<td>0.42 ± 0.23</td>
<td>0.35 ± 0.18</td>
</tr>
<tr>
<td>RR+ag</td>
<td>M₂-GFP + M₂-mCh + carbachol</td>
<td>5</td>
<td>0.45 ± 0.2</td>
<td>0.32 ± 0.19</td>
</tr>
</tbody>
</table>

3.3.5.3 Evidence for GG oligomerization in live cells

GFP-Gα, mCh-Gα, wild-type Gβ, and wild-type Gγ subunits were co-expressed in CHO cells to probe the presence of G protein-G protein oligomerization in live cells using dcFCS. Similar to M₂ receptors, significant cross-correlations were detected between differently labeled Gα subunits. In summary, among a total of 14 cells examined, an average fraction of 47±18% GFP-G and 33±15% mCh-G were found co-diffusing with the other color species. In conjunction with the previously observed coupling between mCh-Gα and GFP-Gβ in the purified samples, it can be inferred that heterotrimeric G proteins form, at least partially, stable oligomers in the cell. The size of the G-protein oligomers will be discussed in detail in Chapter 4.

To investigate the effect of receptor activation on the oligomerization of the G proteins we co-expressed wild-type (unlabeled) M₂ receptors with GFP-G proteins and mCh-G proteins in CHO cells (Fig. 16). Without adding an agonist to the cell culture, significant fcd were found between GFP-G proteins (42±12%) and mCh-G proteins (29±17%). We then added saturating amounts of agonist (10mM carbachol) to the Petri-dish in situ. After incubating for 2min, further dcFCS measurements were taken, and the fcd dropped considerably, to 29±22% of GFP-G proteins and 14±11% mCh-G proteins. Conversely, if the receptors were not co-expressed with the G proteins, the fraction of co-diffusing species was independent of the agonist binding.
Figure 3-20. Representative dfCFS measurement of GFP-G protein and mCh-G protein in live CHO cells, showing significant fractions of co-diffusion (Panel A). The co-diffusion was interpreted as evidence for G protein oligomers. The fractions of co-diffusion remained significant with the presence of either inactive receptor (Panel B) or receptor agonist (Panel C) but was substantially decreased with the presence of active receptors (Panel D).

The dfCFS results of the co-expressed GFP-G proteins and mCh-G proteins are summarized in Table 3-4. It was concluded that, like the receptors, the G proteins exist as oligomers in live CHO cells. In contrast to the M2 receptors whose oligomeric size stays relatively unchanged upon activation by ligand (agonist) binding, the oligomeric size of G proteins decreases upon activation of the receptor and possibly binding of endogenous GTP. The data points to the biological relevance of oligomeric G proteins and receptor-G protein oligomeric complexes for cellular signaling.
Table 3-4. Summary of the dcFCS analysis of G protein-G protein interactions.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sample composition</th>
<th># of cells</th>
<th>fcd green</th>
<th>fcd red</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>Gαi-GFP + Gαi-mCh + Gβ + Gγ</td>
<td>14</td>
<td>0.47 ± 0.18</td>
<td>0.33 ± 0.15</td>
</tr>
<tr>
<td>GG+ag</td>
<td>Gαi-GFP + Gαi-mCh + Gβ + Gγ + carbachol</td>
<td>5</td>
<td>0.47 ± 0.24</td>
<td>0.39 ± 0.2</td>
</tr>
<tr>
<td>(R)GG</td>
<td>M2 + Gαi-GFP + Gαi-mCh + Gβ + Gγ + carbachol</td>
<td>7</td>
<td>0.42 ± 0.12</td>
<td>0.29 ± 0.17</td>
</tr>
<tr>
<td>(R)GG+ag</td>
<td>M2 + Gαi-GFP + Gαi-mCh + Gβ + Gγ + carbachol</td>
<td>17</td>
<td>0.29 ± 0.22</td>
<td>0.14 ± 0.11</td>
</tr>
</tbody>
</table>

3.3.5.4 Pre-signaling RG coupling state in live cells and the effect of agonist

The M2-GFP and the modified mCh-G protein (mutation GaiN270D, Section 3.3.2.6) were co-expressed in live CHO cells to probe the interaction between receptors and G proteins in live cells. When an agonist was introduced, the incubation time was minimized to ~2 min, and only dcFCS measurements taken within ~100 seconds immediately after were analyzed, to reduce the impact of G proteins decoupling from receptors upon binding GTP.

Both M2-GFP with mCh-G protein and M2-mCh with GFP-G protein were tested, and no difference in cross-correlation was found between the different labeling schemes. Among a total of 17 cells measured in the absence of agonist, the fcd values were 25±20% for the M2 receptors and 18±13% for G proteins. After the addition of agonist (10mM carbachol), among a total of 19 cells, the fcd values approximately doubled, to 52±35% for the M2 receptors and 38±18% for the G proteins. The significant increase of the coupled receptor-G protein complexes upon the introduction of agonist indicates that G proteins can be recruited much more efficiently by the activated receptors than the inactive ones. This result supported the scenario in which the majority of receptors and G proteins are not pre-coupled in the absence of agonist, and coincided well with the dcFCS measurements performed on extracted and purified receptor-G protein complexes solubilized in detergent micelles (Section 3.3.2.6).
Figure 3.21. Without mutation GoiN270D, wild-type G protein did not show positive cross-correlation with receptors upon addition of agonist, possibly due to the fast detachment of the activated G proteins from the activated receptors after hydrolyzing endogenous GTP.

Previous FRET results pointed to a major role of a pre-coupled receptor-G protein complex in the fast receptor signaling process [63, 66], but recent studies [13, 54] have disputed that conclusion, and argued in favor for the scenario of G protein recruitment from close vicinity. Our studies supported the latter view. FRET measurements without enough time resolution are solely based on interpreting the FRET efficiency as distances between fluorophores, and thus incapable of differentiating a permanently coupled complex from a transiently interacting complex. Our dcFCS measurements supplement the FRET assays by detecting the true coupling at microsecond to second time scales in the form of co-diffusion.
Figure 3-22. Representative dcFCS measurement of M₂-GFP and mCh-G protein in live cells, showing the nearly absent cross-correlation in the basal state (Panel A, “RG”), and a significant fraction of cross-correlation induced by the activation of the receptor after adding the agonist (Panel B, “RG+ag”).

Considering the abovementioned dcFCS data, which shows that both the M₂ receptors and the G proteins exist and perhaps function as oligomers, we next used the agonist-induced receptor-G protein coupling interaction to further investigate the underlying pathways for cooperativity between receptors. An orthosteric defective mutant of the M₂ receptor, namely M₂D103A [43, 67], was introduced by mutating the Asp at location 103 to Ala. The Asp103, located on the transmembrane segment 3 (TM3), serves as the counter-ion to orient the ligand in the binding site. The mutation has been shown to block the receptor’s ability to bind orthosteric ligands, such as QNB (3-Quinuclidinyl benzilate, an antagonist), and carbachol (an agonist).

The mCh-labeled mutant receptor, M₂D103A-mCh, was co-expressed with GFP-G protein either with or without the wild-type (WT) unlabeled M₂ receptor. When the WT receptors were not present, adding the agonist carbachol and incubating for 2 min did not result in significant fcd between the mutant M₂ and the G protein, with 22±10% of the mCh-M₂D103A receptor and 19±4% of the GFP-G protein showing co-diffusion (Figure 3-23, Panel A). Based on our previous observations (Figure 3-22), low cross-correlations were expected for this case because the orthosteric defective mutant receptors should always be in the basal state regardless of the presence of agonist, and thus have a low
probability to form complexes with G proteins. In contrast, when the wild-type M\(_2\) was present, significantly higher fractions of the mutant receptors (55±37\%) and, to a smaller extent, of the G proteins (27±14\%) were found to be co-diffusing with each other (Figure 3-23, Panel B). The increased fcd pointed to a picture of a cooperative interaction between receptor protomers inside the same M\(_2\) oligomer, allowing the mutant receptor to be recruited by the G protein via contacts between the receptor and/or G protein oligomers. Further studies of oligomeric cooperativity are currently underway in the Gradinaru lab.

![Figure 3-23](image)

**Figure 3-23.** Representative dcFCS measurements on the cooperativity-related coupling interaction between the mutant M\(_2\) receptor M\(_2\)D103A with G protein in the absence or presence of the activated wild type M\(_2\). (A) In the absence of wild type M\(_2\), the orthosteric defective mutant mCh-M\(_2\)D103A showed only basal activity to couple to G proteins despite the addition of agonist. (B) With the presence of wild type M\(_2\), the orthosteric defective mutant mCh-M\(_2\)D103A is able to couple to the GFP-G protein after the addition of agonist.

The dcFCS results of the co-expressed M\(_2\) receptors and G proteins are summarized in Table 3-5. The M\(_2\) receptors and G proteins were found to be separated before the introduction of agonist. The agonist-induced coupling of G proteins to the M\(_2\) receptors was then employed as an indicator of receptor activation, and the cooperative interaction among monomeric receptors inside oligomers was explored, revealing a functional role of oligomerization in the M\(_2\) signaling process.
Table 3-5. Summary of the dcFCS analysis of the receptor-G protein interactions.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sample composition</th>
<th># of cells</th>
<th>fcd green</th>
<th>fcd red</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG</td>
<td>M₂-GFP + GαiND-mCh (or M₂-mCh + GαiND-GFP)</td>
<td>19</td>
<td>0.25 ± 0.2</td>
<td>0.18 ± 0.13</td>
</tr>
<tr>
<td>RG+ag</td>
<td>M₂-GFP + GαiND-mCh (or M₂-mCh + GαiND-GFP) + carbachol</td>
<td>16</td>
<td>0.52 ± 0.35</td>
<td>0.38 ± 0.18</td>
</tr>
<tr>
<td>RDA(R)G</td>
<td>M₂D103A-mCh + M₂ + GαiND-GFP</td>
<td>4</td>
<td>0.24 ± 0.16</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td>RDA(R)G+ag</td>
<td>M₂D103A-mCh + M₂ + GαiND-GFP + carbachol</td>
<td>4</td>
<td>0.55 ± 0.37</td>
<td>0.27 ± 0.14</td>
</tr>
<tr>
<td>RDAG</td>
<td>M₂D103A-mCh + GαiND-GFP</td>
<td>3</td>
<td>0.13 ± 0.04</td>
<td>0.22 ± 0.13</td>
</tr>
<tr>
<td>RDAG+ag</td>
<td>M₂D103A-mCh + GαiND-GFP + carbachol</td>
<td>4</td>
<td>0.19 ± 0.04</td>
<td>0.22 ± 0.1</td>
</tr>
</tbody>
</table>

3.3.5.5 Co-diffusion was not an artifact of crowding

For all live-cell FCS measurements, local densities of the fluorescently labeled proteins on the cellular membrane were estimated based on the FCS fitting parameters \((G(0), s, w₀)\) according to Equation 3.10:

\[
c = \frac{\langle N \rangle}{\text{Area}} = \frac{1}{G(0)} \cdot \frac{1}{\pi s w₀^2}
\]  
(Equation 3.10)

where the detection volume width parameter \(w₀\) and ellipticity factor \(s\) were determined from single color FCS calibration measurements with fluorescent dyes (Chapter 2.1.1, Equation 2.4) for measurements performed on the side membrane; \(s\) was fixed to 1 for the measurements performed on the bottom/top membrane.

The estimated local densities of receptors and G proteins were at the low end for molecular crowding. Considering a typical diffraction-limited confocal detection spot with width \(w₀ \sim 250\,\text{nm}\), the 2D Gaussian effective detection area can be estimated to be \(\sim 0.2 \,\mu \text{m}^2\) at the bottom/top membrane. For the live-cell measurements previously mentioned in this chapter, the concentrations of fluorophore-tagged membrane proteins typically fell in the range of \(10–5000 \,\text{molecules/\mu m}^2\). More specifically, MP-GFP was found to be expressed at a density of \(10–200 \,\text{particles/\mu m}^2\), M₂ receptor was at \(10–1000 \,\text{particles/\mu m}^2\), and G protein was at \(10–2000 \,\text{particles/\mu m}^2\). These levels of expression...
are similar to the reported concentrations from FCS studies of GPCRs [59] and other integral membrane proteins [68, 69]. The average intermolecular distance under the density of 5000 molecules/µm² was estimated to be 14nm, significantly larger than the dimensions of the crystal structures of receptors and G proteins, which are on the scale of ~3nm, and thus disfavoring frequent non-specific interactions.

**Figure 3-24.** Scatter plot and linear regression of $f_{cd}$ vs. $\langle N \rangle$ (the fraction of co-diffusion vs. the estimated number of fluorophores in detection volume) for (A) receptor-containing samples and (B) G protein-containing samples. The slope of linear regression was found to be $(-1.1\pm0.8)\times10^{-3}$ for receptor-containing samples and $(9.2\pm28)\times10^{-5}$ for G protein-containing samples. The p-values for testing the linear regression null hypothesis that the slope equals zero is 0.1 for the receptors and 0.75 for the G proteins, indicating weak evidence for a linear relation between $f_{cd}$ and concentration for the receptors and no evidence for a linear relation between $f_{cd}$ and concentration for the G proteins.

Furthermore, the $f_{cd}$ values were plotted against the concentration of fluorophores for the receptor samples and G protein samples respectively (Figure 3-24). Across the range of the expression levels, there was weak evidence for a negative correlation between the $f_{cd}$ of receptors and their local density at the membrane (Figure 3-24, A). In addition, there was no evidence for an apparent correlation between the $f_{cd}$ of G proteins and their local density at the membrane (Figure 3-24, B). Both of the results indicate that the coupled
complexes identified by the dcFCS technique were not stochastic artifacts as a result of crowding.

3.4 Conclusions and Future Directions

The dcFCS technique, as a major extension to the FCS technique, proved to be a powerful and robust tool in studying bi-molecular interactions both in detergent micelles and in live cells. In the current project, dcFCS was employed to dissect all the possible coupling interactions within the M2 receptor signaling complex – receptor with receptor, G protein with G protein, and receptor with G protein.

Significant fractions of co-diffusion between GFP-M2 and mCh-M2 receptors were detected both in detergent micelles (Figure 3-9) and in live cells (Figure 3-19), regardless of the presence of receptor agonist. It was concluded that the M2 receptors exist as oligomers with a size of at least two. The size of the oligomer likely remains unchanged upon activation, yielding statistically unchanged fractions of co-diffusion.

Significant fractions of co-diffusion between GFP-G proteins and mCh-G proteins were detected both in detergent micelles (Figure 3-10) and in live cells (Figure 3-20). In the presence of activated M2 receptor, the fraction of co-diffusion dropped to near zero in live cells (Figure 3-20). It was concluded that when not interacting with receptors, the G proteins exist as oligomers with a size of at least two; after interacting with activated receptors in live cells, the oligomeric size of the G proteins likely decreases, yielding lower fractions of co-diffusion. Our results suggested a functional role of G protein oligomers – whereby oligomeric G proteins may split into smaller oligomers or even monomers to achieve high signal transduction efficiency.

Low fractions of co-diffusion between basal state M2 receptors and G proteins were detected both in detergent micelles (Figure 3-11) and in live cells (Figure 3-22). The fractions of co-diffusion significantly increased between activated M2 receptors and G proteins both in detergent micelles (Figure 3-11) and in live cells (Figure 3-22). Together these results supported the recruitment transient coupling scenario of the receptor-G protein interaction in which G proteins are only recruited and transiently interact with the
activated receptor. Under such a scenario, the G proteins have to be available in close vicinity of the receptor to facilitate fast response, and the rate of response is diffusion limited.

Cooperative behaviors were observed in live cells within the oligomeric receptors containing both wild-type M2 receptors and orthosteric defective mutant M2 receptors. With the presence of activated wild-type M2 receptors, the orthosteric defective mutant M2 receptors regained the ability to couple to G proteins (Figure 3-23).

Most of the estimations of oligomeric sizes in the current chapter are qualitative without single molecule resolution (i.e. behaviors of all the molecules that pass through the detection volume are averaged). Quantitative estimations of the oligomeric sizes of receptors and G proteins were carried out with single-molecule photobleaching stoichiometry (smPB) and summarized in Chapter 4. Sub-ensemble behaviors of individual receptor / G protein complexes at the membrane of live cells were carried out with single particle tracking (SPT) technique and summarized in Chapter 5.

Furthermore, dcFCS measurements with two-photon excitation (TPE) will be employed to resolve the receptor-G protein interaction in live cells with higher spatial resolution and reduced background noise.

3.5 References


4 SmPB on Receptor and G Protein Oligomers

4.1 Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of transmembrane proteins, with almost 4% of the protein-encoding portion of the human genome producing nearly 1,000 receptors [1]. GPCRs detect a remarkably diverse set of extracellular stimuli, from photons of light to calcium, small organic molecules such as neurotransmitters and odorants, peptides, glycoproteins, and phospholipids. They occur in all organs and most tissues throughout the body [2, 3], and they regulate various intracellular processes [4]. They are implicated in many if not most diseases, and they are the targets of almost one third of prescribed drugs [5]. In short, they are very important proteins.

All GPCRs serve as transducers of the signal between the extracellular stimulus and intracellular mediators, particularly G proteins and arrestins [6]. Binding of an agonist generally is believed to initiate the process by promoting coupling of the receptor to a GDP-bound G protein [7-10]. That in turn causes the release of GDP and the formation of a stable complex between a nucleotide-free G protein and an agonist-bound receptor [11, 12]. Guanosine triphosphate (GTP) then binds to the site vacated by GDP, causing the complex to dissociate into a GTP-bound G protein and an uncoupled receptor with reduced affinity for the agonist [8, 9]. The release of G proteins occupied by GTP, or by a non-hydrolyzable analogue such as GTPγS, leads to downstream signaling [13]. This general understanding has fostered a view of receptors as simple on-off switches, but that now seems simplistic. More recent evidence for multiple active states of the receptor and preorganized signaling units has pointed to greater complexity and prompted a reexamination of prevailing theories and models [14].

Adding to this complexity, GTP-induced changes in the affinity of agonists for muscarinic receptors in myocardial membranes were found to differ from those predicted for transient complexes of monomeric receptors and G proteins [9]. In a comparison of purified M2 muscarinic receptors reconstituted as monomers in size-selective nanodiscs and as tetramers in phospholipid vesicles, it was demonstrated that only the latter
reproduce the effects of guanylyl nucleotides on the receptor in sarcolemmal preparations [15]. Moreover, cooperative interactions between the constituent protomers of an oligomer can account for ligand-binding properties that otherwise are difficult to explain [8], and they have been shown to underlie the functioning of GPCRs of Family C such as the metabotropic glutamate and GABA receptors [16]. Dimers or larger oligomers of the M$_2$ and M$_3$ muscarinic receptors have been identified directly through biochemical approaches, such as co-immunoprecipitation [17] and chemical cross-linking [18], and in biophysical studies based on bioluminescence and Förster resonance energy transfer (BRET [19] and FRET [20]).

The role served by oligomers of GPCRs has been a subject of much debate. The controversy is due in part to questions regarding the design and interpretation of studies based on BRET [21, 22]. Also, measurements of single-molecule fluorescence have identified a preponderance of monomers in some studies [23, 24] and of dimers in others [25, 26]. Such differences in the oligomeric state may be due to differences among different receptors, in the preparation of samples, or in the interpretation of data. FRET-based attempts to estimate the oligomeric size of a GPCR upon activation by agonists similarly have led to mixed results, with an increase in one case [27] and little or no change in others [20, 28]. Challenges typically faced in such experiments include uncertainty over what fraction of the total number of receptors is being measured and the difficulty of detecting and accounting for mixtures of oligomers of different size.

Approaches based on single-molecule fluorescence, such as single-molecule photobleaching (smPB), provide a direct measure of the oligomeric size of individual particles and therefore give access to the distribution of oligomeric sizes across the full ensemble. Such experiments typically involve the immobilization of individual protein complexes in which each protomer is tagged with a single fluorophore. The oligomeric size of each complex then is inferred from the number of step-wise drops in the trace of fluorescence intensity over time [29-31]. In this study, we characterized the oligomeric states of the M$_2$ receptor and the G$_{i1}$ protein, both separately and coupled together in a complex. To obtain the required probes, enhanced green fluorescent protein (GFP) was fused to the N-terminus of the human M$_2$ muscarinic cholinergic receptor, inserted in the
Ga1-subunit, or fused to the N-terminus of the Gγ2-subunit. The signal was calibrated using fused multimers of GFP of known oligomeric size, purified to homogeneity.

Distributions of the number of photobleaching steps were acquired for the receptor and Gi1 at different stages of the signaling process, which were attained by treatment of the immobilized particles with agonists, antagonists, and guanylyl nucleotides. The results of in vitro photobleaching measurements were complemented by live-cell dual-color fluorescence correlation spectroscopy (dcFCS) and quantitative FRET, which were used to identify and characterize oligomers of GFP- and mCherry-tagged Gi1 in live CHO cells. The spectroscopic data describe a supramolecular complex comprising an oligomer of the M2 receptor on the one hand and an oligomer of Gi1 on the other.

4.2 Materials and Methods

4.2.1 Expression and purification of multiplexed GFP

For the purpose of calibrating smPB counting statistics, standard multiplexes of GFP were prepared in which 1, 2, and 4 units of the fluorophore were fused head-to-tail. Each standard was fused at the N-terminus to a sequence corresponding to residues 1–31 of Ga1 (MP) and at the C-terminus to hexahistidine (His6). The amino terminus of Ga1 contains the sites of myristoylation and palmitoylation, and it was introduced to localize GFP×j at the membrane of E. coli in a manner similar to that of Ga1 or Gi1 in CHO cells. Each standard was expressed in E. coli and purified via the His6 tag by chelating chromatography on Ni²⁺-NTA. Samples were recovered from the column by elution in the presence of imidazole (250 mM), which subsequently was removed by repeated concentration and dilution with PBS (Sigma-Aldrich) in a Centricon-50 concentrator. The homogeneity and multimeric nature of each preparation was confirmed by electrophoresis and western blotting, and the results from two batches of each standard are shown in Figure 4-1.
Figure 4-1. Design and characterization of GFP×j. (A) Single and multiplexed GFP×j were tagged at the N-terminus with a membrane-anchoring sequence corresponding to residues 1–31 of Gαi1 (coded as “MP”) and at the C-terminus with hexahistidine (His6). (B) Western blots of purified His6-GFP×1, His6-GFP×2, and His6-GFP×4. Each lane of the gel was loaded with ~ 0.1 nmol of the protein, and the molecular weights of the constructs correspond with the expected values.

4.2.2 Preparation of receptor samples and G protein samples

The preparation protocol of M2 receptors and G proteins follows Chapter 3.2.1.1. Substitutions, insertions, and deletions of bases were performed by site-directed mutagenesis (Quick-Change, Agilent Technologies). PAGE-purified primers were obtained from Integrated DNA Technologies (IDT). Constructs for expression in CHO cells were prepared in pcDNA3.1; those for expression in Sf9 cells were prepared in BacN-Blue (Life Technologies). The human M2 muscarinic receptor was fused at either the N-terminus [1] or the C-terminus to green fluorescent protein (GFP). Fluorophore-tagged Gαi1 was constructed by inserting the sequence coding for GFP or mCherry (mCh) after position 91 using the polymerase chain reaction and a megaprimer [2] (GFP-Gαi1, mCh-Gαi1). Wild-type human Gβ1 and human Gγ2 proteins with His6 at the N-terminus (His6-Gβ1, His6-Gγ2) were purchased from the cDNA Resource Center (www.cdna.org). After extraction and solubilization, all constructs were purified using chelating chemistry of the His6-tag on Ni2+-NTA columns.
4.2.3 Functionality of tagged G proteins and receptors

The functionality of the purified modified M<sub>2</sub> receptors was confirmed through radioligand binding. The affinity of <sup>3</sup>Hmethylscopolamine ([<sup>3</sup>H]NMS) was indistinguishable in assays on the wild-type receptor and both fluorophore-tagged mutants (log \( K = -8.01 \pm 0.12 \)). It was therefore concluded that the fused fluorescent protein did not affect the ability of the receptor to bind the radiolabeled antagonist, as is also reported previously [20].

Human Gα<sub>i1</sub> and heterotrimeric Gα<sub>i1</sub>β<sub>1</sub>γ<sub>2</sub> were extracted in GDP-free form from S<sub>f9</sub> cells as described in Chapter 4.2.1. Gα<sub>i1</sub> was modified by the insertion of hexahistidine (His<sub>6</sub>), GFP, mCherry (mCh), or combinations thereof between positions 91 and 92 to form His<sub>6</sub>-Gα<sub>i1</sub>, GFP-Gα<sub>i1</sub>, His<sub>6</sub>-GFP-Gα<sub>i1</sub>, mCh-Gα<sub>i1</sub>, and His<sub>6</sub>-mCh-Gα<sub>i1</sub>. The functionality of the modified G proteins were confirmed through two independent assays, namely radioligand ([<sup>35</sup>S]GTP<sub>γ</sub>S) binding, and fluorescence correlation spectroscopy.

The insertion of a fluorescent protein with or without His<sub>6</sub> did not interfere with the GTP binding ability of the G protein. In binding studies on His<sub>6</sub>-tagged Gα<sub>i1</sub> and Gα<sub>i1</sub>β<sub>1</sub>γ<sub>2</sub>, the affinity of [<sup>35</sup>S]GTP<sub>γ</sub>S was similar or the same irrespective of the presence of GFP (His<sub>6</sub>-Gα<sub>i1</sub>, log \( K = -5.66 \); His<sub>6</sub>-GFP-Gα<sub>i1</sub>, log \( K = -6.26 \)) or of Gβγ (GFP-Gα<sub>i1</sub>β<sub>1</sub>-His<sub>6</sub>-γ<sub>2</sub>, log \( K = -6.01 \)). This indicated that the primary function of the G protein was preserved after the modifications.

The insertion of a fluorescent protein with or without His<sub>6</sub> did not disrupt the formation of holo-G<sub>i1</sub> from Gα<sub>i1</sub> and Gβγ. When mCh-Gα<sub>i1</sub> and GFP-Gβ<sub>1</sub>γ<sub>2</sub> were co-expressed in CHO cells and monitored by dcFCS (Figure 3-18, C), the amplitudes of the auto- and cross-correlation curves indicated that 91% of the limiting species—in this case, mCh-Gα<sub>i1</sub>—migrated as a heterotrimer. There was little or no cross-correlation with a negative control in which mCh-Gα<sub>i1</sub> was co-expressed with a fusion protein comprising the first 30 amino acids of Gα<sub>i1</sub> (MP) and GFP (i.e., MP-GFP) (Figure 3-18, F). The MP sequence contains the sites of myristoylation and palmitoylation in Gα<sub>i1</sub> [32] and is responsible for its localization at the plasma membrane.
4.2.4 Total Internal Reflection Fluorescence Microscope

The smPB experiments were performed on the Total Internal Reflection Fluorescence (TIRF) Microscope in the Gradinaru Lab. The details of the setup are provided in Chapter 2.3.1 [33]. In brief, samples were excited at 473 nm using a diode-pumped solid-state laser (Cobolt Blue, Colbolt AB, Sweden). The excitation intensity was controlled by an acousto-optic tunable filter (TF625-350-2-11-BR1A, Gooch & Housego). The excitation light was reflected by a dichroic mirror (FF495-Di02, Semrock) and illuminated the sample through an oil-immersion objective (1.45NA/60× Plan-Apochromat, Olympus, USA). The green fluorescence emission was collected using the same objective and transmitted through the dichroic mirror. Scattered light was filtered out using a long-pass (LP-488-RS, Semrock) and a band-pass filter (HQ512/25, Chroma). Samples were imaged with an electron-multiplied charge-coupled device (EMCCD, DU-897BV, Andor Technology) cooled to −70 °C. The area of detection was approximately 50 μm × 50 μm, and a sequence of 500 frames (movie) was acquired with an exposure time of 33 ms/frame. About 20 different areas of the sample were imaged for each chamber, and each area contained 50–100 identifiable fluorescent spots (particles).

4.2.5 Construction and characterization of flow chambers

Flow chambers were constructed to accommodate for the requirements of the single molecule pull-down technique outlined in Section 4.2.6.

Figure 4-2. Schematic of the flow chamber. The volume of the chamber created with the protocol stated in this chapter is typically ~50 μL. The inlet/outlet holes allow for convenient addition/removal of new samples/solutions to the immobilization surface. A piece of Kimwipe disposable wiper is usually used at the outlet hole to facilitate the liquid flow.
3”×1” microscope glass slides (VWR, CA48300-025) were drilled (Dremel Model 220-01, speed setting 3) with 1/16” diamond bits (Dremel #11618RB) to create a pair of inlet and outlet holes that are separated by approximately 0.8”. The slides and No. 1 coverslips (VWR, CA48366-089-1) were then soaked in water in a clean glass container for 12 h, and washed in boiling water in a microwave oven for 10 min, then rinsed with distilled water and swiped 5 times through the non-oxidizing tip of a propane-fueled flame for instant drying.

To activate the glass surfaces, the flame-dried slides and coverslips were sonicated for 30 min in each of three consecutive baths: 1.0 M KOH, 0.5% Hellmanex solution (Sigma-Aldrich, Z805939-1EA), 99.9% HPLC-grade acetone (Sigma-Aldrich, 270725-1L), and 1.0 M KOH. Each surface then was rinsed with distilled water and swiped 5 times through the non-oxidizing tip of a propane-fueled flame for instant drying.

To coat polyethylene glycol (PEG) onto the activated surfaces, the slides and the coverslips were sonicated for 10 min in 99.9% HPLC-grade methanol (Sigma-Aldrich, 34860-1L-R) and transferred to a beaker containing a 100:5:1 mixture of methanol, glacial acetic acid (Sigma-Aldrich, 320099-2.5L), and 3-aminopropyl-triethoxysilane (ATPES, Sigma-Aldrich, A3648-500ML), where they were sonicated in the dark for 15 min at room temperature. The surfaces then were washed in methanol and dried under argon. An aliquot (45 μL) of a freshly made solution of mPEG (72 mg, mPEG-SVA, MW 5000, Laysan Bio) and biotin-PEG (3 mg, Biotin-PEG-SVA, MW 5000, Laysan Bio) in 360 μL of 10 mM sodium bicarbonate (pH 8.5) was sandwiched between two dried coverslips to ensure uniform surface passivation on both slips. PEG-treated surfaces were incubated for 8 h at room temperature in a custom-made humidified storage unit.

After 8 h of incubation with PEG, the two surfaces were separated, washed with deionized water, and dried under argon. Two adjacent strips of double-sided tape (3M) were placed between the coverslip and the slide, as shown in Figure 4-2, to create a gap of 3–5 mm between the strips. The remaining two edges were sealed with epoxy-super glue (ITW Devcon, cat. No. 6245), and the assembled unit was stored in a vacuum.
chamber. The passivation efficiency of these surfaces typically remained optimal for up to two weeks at room temperature.

4.2.6 Immobilization of single molecules

The immobilization protocol of single molecules was adopted from the single molecule pull down technique published by the Ha Group [34].

In a PEGylated flow chamber prepared as described above, a solution (50 µL) of streptavidin (4.0 µg/L, Life Technologies, cat. No. 43-4302) in PBS was flowed through and incubated for 15 min. The chamber then was washed by flowing through four volumes of PBS buffer (200 µL). A solution (50 µL) of biotinylated anti-His\textsubscript{6} antibody (10 µg/L, Abcam, ab27025) in PBS was introduced into the washed chamber and incubated for 10 min at room temperature, after which the chamber was flushed with four volumes of the appropriate sample buffer. An aliquot (50 µL) of the His\textsubscript{6}-tagged complex (1–10 nM) was introduced into the chamber and incubated for 15 min. Any unbound sample then was removed by washing with four volumes (200 µL) of sample buffer.

In this protocol, the amount of streptavidin was the limiting factor dictating the surface density of the immobilized molecules—both the biotinylated PEG and the His\textsubscript{6}-tagged sample were in excess. The concentration of streptavidin can be quantified more accurately than both PEG (in the form of powder) and the sample protein (purified from cells). Using streptavidin as the limiting factor proved to consistently yield optimal surface coverage, avoiding the difficulty in fine-tuning the biotin-PEG:PEG ratio and the uncertainty in the concentration of His\textsubscript{6}-tagged sample. In addition, with excessive biotin-PEG sites available, it was possible to increase the surface density of the immobilized molecules when necessary.
Figure 4-3. Illustration of the single molecule immobilization scheme. Upon the completion of the immobilization procedure, the surface of the flow chamber is coated with four layers: (1) mPEG and biotinylated PEG, (2) streptavidin, (3) biotinylated anti-His6 antibody, and (4) His6-tagged sample molecule.

The fluorescent impurities induced in each layer were monitored with the TIRF microscope. As shown in Figure 4-4, the density of the impurities was lower than 5 per frame (i.e. 0.002/µm²), which is much lower than the density of the His6-tagged samples (typically 50~250 per frame, or 0.02~0.1/µm²).

Figure 4-4. TIRF images of the chamber are shown after treatment with PEG-biotin (A), PEG-biotin plus streptavidin (B), PEG-Biotin plus streptavidin and biotin-anti-His6 (C), and PEG-biotin plus streptavidin, biotin-anti-His6, and GFP-M2-His6 (D). The bars in panels A–D indicate 5 µm.
4.2.7 Analysis of TIRF image stacks

In the TIRF microscope, the Gaussian excitation laser beam passed at a large angle through the objective to create an evanescent and non-uniform sample illumination. To account for this spatial inhomogeneity, TIRF images were recorded for a droplet of dye (0.5 μM, Rhodamine 6G) before each series of single-particle acquisitions. After subtraction of the background, a flatness matrix was computed by dividing the intensity of each pixel in the image by that of the brightest pixel. Each frame of subsequent recordings was corrected by dividing the raw data matrix by the flatness matrix to homogenize the intensity across the entire TIRF image of immobilized fluorophores.

Figure 4-5. Outline of the TIRF image stack analysis scheme. Single spots were identified in each frame (A), and the signal from the pixels corresponding to each spot was monitored over time. (B) Each intensity-time trace (blue line) was analyzed according to a change-point algorithm to identify bona fide photobleaching steps (black line). Changes in intensity that were below the threshold of the step-function, and therefore were disregarded, are shown by the red line. (C) Identified photobleaching steps from all recorded molecules within a sample were used to build a histogram of the number of photobleaching steps per particle, which can be fit to a binomial distribution, for which different n values (maximum number of steps) can be chosen (solid lines, different colors).

After correction for flatness and subtraction of the background, the stack of 500 images was processed to identify single particles (spots) and to extract intensity-time trajectories. Spots with a diameter of 3 pixels (i.e., ~300 nm) were identified in a two-step procedure based on contrast and brightness, with a brightness threshold of 0.88 (only particles brighter than 88% of all the pixels were qualified for analysis). To obtain the center
position, the width, and the amplitude of each spot (molecule), a model based on 2D-Gaussian maximum-likelihood estimation (MLE) was fitted to the data \cite{35}. The analysis was applied to each successive frame in order not to exclude particles that were initially in the dark state (i.e., blinking). The locations \((x, y)\) of the \(3 \times 3\) pixel areas corresponding to the identified spots were indexed, and all overlapping spots within a distance of 9 pixels were removed to avoid analyzing separate particles tethered too close to each other (Figure 4-5, A). Intensity-time trajectories then were stitched together frame by frame to create a single intensity-time trajectory for each identified spot (Figure 4-5, B).

### 4.2.8 Photobleaching analysis of the intensity-time trajectories

#### 4.2.8.1 Change-point analysis

Each intensity-time trace was processed to evaluate the number of photobleaching (PB) steps and the intensity associated with each step (Figure 4-5). Transitions in the single-molecule trajectories were identified by means of a change-point algorithm based on the principles laid out by Watkins and Haw \cite{36} (Figure 4-5). The algorithm does not require any prior knowledge of the signal dynamics or the noise statistics. It first identifies putative change-points and then calculates the likelihood that each of them is a true change-point according to a user-defined criterion. The change-point algorithm was implemented in the Gradinaru Lab by Zhenfu Zhang.

For any intensity-time vector \(i(t)\), a cumulative-sum trajectory, \(CS(t)\), is calculated according to the formula:

\[
CS(t) = \sum_{t'=1}^{t} (i(t') - <i>)
\]

(Equation 4.1)

where \(<i>\) is the time-average of \(i(t)\). In the absence of any transition in the raw intensity, the cumulative sum fluctuates around zero. If a transition occurs, \(CS(t)\) changes from low to high or from high to low values, depending on the direction of the change in the original signal. Change-points are assigned at the time points where \(|CS(t)|\) has local maxima.
Next, these change-points are assessed against a user-defined confidence level (e.g., \( p = 90\% \)). A statistical \( t \)-test is performed on the intensity data points before and after each putative change-point. If the output is higher than the logarithm of the odds, \( i.e., \log((1 - p)/p) \), the change-point is considered significant; otherwise, it is designated as noise and disregarded. In effect, the photobleaching intensity traces are fitted by a trajectory with discrete intensity levels interspersed with stepwise jumps (Figure 4-5 B).

For the purpose of this study, reversible change-points (photoblinking) were discarded from analysis. Photoblinking was defined as a change in intensity that exceeded 300 counts-per-second (cps), lasted for at least four successive frames (\( i.e., 4 \times 33\text{ms} = 132\text{ms} \)), and then returned to within 10% of the initial level. Such blinking events are typically downward, at the benchmark of 300 cps, corresponding to \( \sim \)20% of the average photobleaching step size observed with the GFP\(_{\text{xj}}\) standards (Section 4.3.3 and Figure 4-9). Intensity values during the blinking event were replaced by the value immediately prior to the blinking event.

Many fluorophores are known to have ‘dim’ fluorescent states in which the molecular brightness is less than the typical value [37]. To prevent counting the dim states as a false positive photobleaching step, an intensity threshold of 500 cps, or \( \sim 30\% \) of the average photobleaching step-size of GFP\(_{\text{xj}}\) (Section 4.3.3 and Figure 4-9) was introduced. Any change-points with intensity drop less than the dim state threshold were discarded. The change-points before and after applying the dim-state filter are illustrated by the red and black traces respectively, in Figure 4-5 B.

### 4.2.8.2 Photobleaching histograms and statistical analysis

Each accepted downward step in a photo-bleaching trace was indexed from the bottom up; that is, the last step before complete photobleaching was termed step 1, the penultimate step was termed step 2, and so on (Figure 4-5, B). The distribution of the number of PB steps per particle was presented as a normalized histogram (Figure 4-5, C), which was analyzed in terms of the binomial distribution (Equation 4.4). This presupposes that each particle or oligomer consists of \( n \) independent subunits, each of
which has an equal probability of excitation and detection, $p$. Importantly, oligomers were assumed to be homogeneous and non-interconverting.

Further, it was assumed throughout that the immobilized complexes of GFP-tagged protomers are homomeric. That is likely to be true for the $M_2$ receptor; in the case of $G_{i1}$, however, the endogenous G proteins of $Sf9$ cells may give rise to a heteromeric complex containing GFP-tagged and non-fluorescent protomers. This possibility was addressed in part by analyses in terms of Equation 4.2, in which the number of excited and detected protomers ($x$) in a hetero-oligomer of size $n$ is determined by the number of excitable protomers ($i$), the probability of exciting and detecting one such protomer ($\alpha$), and the probability of having a dark protomer ($\beta$).

$$f(k; n, \alpha, \beta) = \sum_{i=k}^{n} \binom{n}{i} \alpha^k (1-\alpha)^{i-k} \cdot \binom{n}{n-i} \beta^{n-i} (1-\beta)^i$$  \hspace{1cm} (Equation 4.2)

If all protomers are incorporated randomly into the complex, $\beta$ is equivalent to the mole fraction of dark protomers. When $\beta = 0$, the model collapses to the binomial theorem with the probability of excitation and detection $p = \alpha$. The probability of eventually detecting $k$ protomers on an oligomeric molecule consisting of $n$ protomers is then given by:

$$f(k; n, p) = \frac{n!}{k!(n-k)!} p^k (1-p)^{n-k}$$  \hspace{1cm} (Equation 4.3)

To further discriminate the dim fluorescent states from the true PB steps, we performed statistical analysis on the intensity of PB steps. The distribution of intensities of each indexed PB step and the total intensity observed for the sum of all PB steps ($i.e.$, the initial intensity of each trace) were fit by a generalized extreme value (GEV) model (Equation 4.4) [38].

$$f(I; \mu, \sigma, \xi) = \frac{1}{\sigma} \left(1 + \frac{I - \mu}{\sigma} \cdot \frac{1}{\xi}\right)^{-1} \cdot \left[-\left(1 + \frac{I - \mu}{\sigma} \cdot \frac{1}{\xi}\right)^{-1}\right]$$  \hspace{1cm} (Equation 4.4)
The model described by Equation 4.4 is defined by three parameters that determine the location ($\mu$), shape ($\xi$), and width ($\sigma$) of the peak. The value of $\xi$ describes the behavior of the exponential tail and spans the range from 0 to 1, with 1 corresponding to the narrowest peak with the longest exponential tail. Both the binomial distribution and the GEV distribution were fitted to the data in MATLAB using the function for maximum-likelihood estimation (MLE) with a confidence interval of 95%.

4.3 Experimental Results

4.3.1 DcFCS Evidence of oligomeric receptor and G protein

In our lab, we have shown the existence of oligomeric $M_2$ receptors and $G_1$ proteins using dual-color Fluorescence Correlation Spectroscopy (dcFCS). As is summarized in Chapters 3.3.2 and 3.3.5, significant fractions of co-diffusion was observed between GFP- and mCh-labeled $M_2$ receptors and between GFP- and mCh-labeled $G_1$ proteins two both purified in detergent micelles and expressed in live CHO cells. It was concluded that $M_2$ receptors and $G_1$ proteins are in the form of oligomers with size of at least two. The oligomerization of $M_2$ receptors also displayed cooperative roles during the signaling initiation phase by agonist (Chapter 3.3.5.5), implying the functional role of oligomeric receptors.

![Figure 4-6](image)

**Figure 4-6.** Oligomers of $G_{i1}$ in live cells. GFP-$G_{i1}$ and mCh-$G_{i1}$ were co-expressed together with $G_{i1}$ in CHO cells and monitored with dcFCS. Fluorescence correlation curves are shown.
as scatter plots for the auto-correlations of GFP-Gαi1 (□, green) and mCh-Gαi1 (○, red), and cross-correlation (△, blue). The solid lines represent the best fits to Equation 3.9, and the dotted lines indicate the contribution of diffusing species without the influence of photophysical fluctuations. The fractions of co-diffusing species are shown in the inset, where the negative control (“-ve”) is the fraction of co-diffusion obtained between two independent molecules, MP-GFP and mCh-Gαi1.

Figure 4-6 shows a typical dcFCS measurement of the co-diffusion of G proteins in live CHO cells. The fractions of co-diffusion species were determined to be 48% of GFP-Gi and 52% of mCh-Gi. Considering the estimated fraction of 40–50% non-fluorescent mCherry expressed in live cells [39], the measured fractions of co-diffusion species can therefore be regarded as the lower limit, thus implying the presence of a significant proportion of oligomeric G proteins in CHO cell. The size of these oligomers is relatively difficult to assess in dcFCS studies, but rather straightforward to determine using the smPB approach.

4.3.2 FRET evidence of oligomeric receptor and G protein

Further evidence of oligomeric G proteins in live cells was gathered using FRET measured in spectrally-resolved confocal microscope (LSM710, Zeiss). To that end, GFP-Gα1 and mCh-Gα1 were co-expressed in CHO cells. The cells were illuminated at 488 nm and with an intensity of ~500 W/cm². A typical sample area of 135 μm × 135 μm was imaged using a scanning 5-nm detection window between 495 nm and 640 nm. From the resulting stack of 30 confocal images, the analysis software constructed the fluorescence emission spectrum at each pixel in the imaging area. The full emission spectrum \( F(\lambda) \) from each cell was unmixed into the contributions of the two fluorophores, using the emission spectra of the donor \( F_D(\lambda) \) and of the acceptor \( F_A(\lambda) \):

\[
F(\lambda) = k_D \cdot F_D(\lambda) + k_A \cdot F_A(\lambda)
\]

(Equation 4.5)

where the fitted values \( k_D \) and \( k_A \) are the contributions of donor and acceptor, respectively. The \( k_D \) and \( k_A \) values were then used to calculate the FRET efficiency, \( E \), for each cell.
Equation 4.6

\[ E_{\text{app}} = \left(1 + \frac{k_D}{k_A} \cdot \frac{\Phi_A}{\Phi_D} \cdot \frac{W_A}{W_D}\right)^{-1} \]

where \( \Phi \) is the fluorescence quantum yield (\( \Phi_D = 0.70, \Phi_A = 0.22 \)), and \( W \) is the fraction of the spectral integral (\( W_A/W_D = 1.06 \)).

Figure 4-7. Spectrally resolved confocal imaging of CHO cells co-expressing GFP-\(\alpha_{i1}\) and mCh-\(\alpha_{i1}\). Cells were excited at 488 nm, and the measured emission spectrum from the region of the membrane of a typical cell is shown in the figure (○). The spectrum was unmixed as described in Equation 4.5 to obtain the fitted spectrum (black) and the individual contributions of donor (GFP, green) and acceptor (mCh, red). Images of the cell are shown in the insets, from left to right: emission from the donor upon excitation at 488 nm, emission from the acceptor upon excitation at 543 nm, FRET efficiency. The efficiency was approximated as the ratio of emission intensities corresponding to the acceptor (585–640 nm) and the donor (495–585 nm) from single pixels upon excitation at 488 nm. Distance scale bar, 10 \( \mu \)m; FRET scale bar, 0–100%.

To verify that the estimated FRET efficiency was not a result of non-specific transient interactions due to molecular crowding in the cell, the influence of the local concentration of fluorophores was studied. GFP-\(\alpha_{i1}\) and mCh-\(\alpha_{i1}\) were expressed at different transfection levels to achieve different local concentrations at the membrane. Among 279 cells measured, the acceptor-to-donor ratio, \([A]/[D]\), varied 64-fold and the FRET efficiency increased with \([A]/[D]\) up to a saturation level of 50–75% in a manner that was
independent of the total amount of expressed G protein (see also Fig. 2C in the published manuscript [40]). The densities of molecules on the cellular membrane were estimated to be within the ranges of $1.5 \text{–} 34 \times 10^3 \, \mu m^{-2}$ for GFP-Gαi1 and $1.3 \text{–} 15 \times 10^3 \, \mu m^{-2}$ for mCh-Gαi1. The average intermolecular distance between fluorophores at a concentration of $15 \times 10^3 \, \mu m^{-2}$ is 9 nm, which is 1.9-fold greater than the Förster radius of 4.86 nm for GFP and mCherry (see also the SI section S2 in the published manuscript [40]). Even at an average intermolecular distance of only 7 nm (equivalent to $20 \times 10^3 \, \mu m^{-2}$), the theoretical FRET efficiency of 10 % is markedly lower than the measured $E$ values (50–75 %) at high ratios of acceptor to donor. Essentially the same trends of FRET were obtained in cells co-expressing differently labeled holo-Gi1 proteins (GFP-Gαi1β1γ2 and mCh-Gαi1β1γ2), as well as with the cells co-expressing differently labeled M2 receptors (GFP-M2 and mCh-M2).

**Figure 4-8.** Spectrally resolved confocal imaging of CHO cells co-expressing MP-GFP and mCh-Gαi1. The data were acquired and processed as described for Figure 4-7. Images of the cell are shown in the insets, from left to right: emission from the donor upon excitation at 488 nm, and emission from the acceptor upon excitation at 543 nm. The calculated FRET efficiency was lower than 5% (shown by the unmixed spectra), despite the presence of both GFP and mCh. Distance scale bar is 10 μm.
In addition to the co-expressed GFP- and mCh- labeled proteins that were found to be coupled and with a finite FRET efficiency, negative control measurements were performed on a pair of functionally irrelevant fluorophores: the membrane-bound GFP (MP-GFP) and the mCh-Gαi1. When imaged separately, both fluorophores were found localized at the plasma membrane (Figure 4-8, inset). When the full emission spectrum was collected with the excitation of GFP, the mCh emission signal was significantly weaker than that of the GFP (Figure 4-8). After unmixing the spectra, the calculated values of FRET efficiency from 52 cells were essentially independent of the relative amounts of donor and acceptor, and never exceeded 5% despite increased local concentration ratios of acceptor to donor. This result also confirmed that the FRET observed between the G proteins and between the receptors were not artifacts of non-specific transient interactions.

4.3.3 Photobleaching of multiplexed GFP

To establish the relationship between the number of smPB steps and oligomeric size, the photobleaching properties of fluorophores within multimers of known size were examined using purified standards comprising one, two, or four equivalents of GFP (i.e., GFP_{j}, j = 1, 2, or 4). Sparsely (with a density of 0.10 particles/μm², which corresponds to a mean distance between particles of about 3 μm) immobilized particles of GFP_{j} were irradiated at 473 nm and ~180 W/cm² under a TIRF microscope, and the fluorescence intensities were recorded over time. The step-wise PB transitions displayed by each particle were identified by the change-point algorithm (Section 4.2.8.1), and histograms of the number of PB steps obtained for GFP_{x1}, GFP_{x2}, and GFP_{x4} are shown in Figure 4-9, A–C.

Almost without exception, the maximum number of PB steps was less than or equal to the number of fluorophores within the multiplex. For instance, 97% of the fluorescent spots in images of GFP_{x1} showed a single PB step, and the remaining 3% showed 2 PB steps. Similarly, particles of immobilized GFP_{x2} showed 1 or 2 PB steps, and those of immobilized GFP_{x4} showed 1–4 PB steps; no particle displayed more than 2 or 4 PB steps, respectively. Equivalence between the maximum number of PB steps and the number of fused fluorophores indicates that there was little or no colocalization of GFP_{x1}
or the higher multiplexes. The absence of PB steps in excess of those expected of a single molecule of GFP$_{xj}$ is consistent with the low average density of immobilized particles, which was 0.01–0.10 per μm$^2$ with GFP$_{xj}$ and the various complexes of receptor and G protein in the subsequent measurements.

**Figure 4-9.** Sparsely immobilized particles of GFP$_{xj}$ were excited at 473 nm and an intensity of 180 W/cm$^2$ to produce the histograms of PB steps (green bars) shown for GFP$_{x1}$ (A), GFP$_{x2}$ (B), and GFP$_{x4}$ (C). The grey bars in panels B and C show the best fit to Equation 4.3 ($n = 2$ and 4, respectively). The inset to panel A shows the distribution of intensities defined by the amplitudes of the single steps in the intensity-time traces; the line represents the best fit of Equation 4.3, and the fitted value of $\mu$ is 1,243 ± 52 (Table S3). (D) smPB distributions for GFP$_{x4}$ were also measured at an intensity of ~60 W/cm$^2$ (light green bars) and ~260 W/cm$^2$ (dark green bars).

In accord with the limit on the number of PB steps, the step-histograms are well described by the binomial distribution with $n$ taken as the number of fused GFPs (Equation 4.3, Figure 4-9, B and C). Larger or smaller values of $n$ gave diminished agreement (See also Fig. S3H in the published manuscript [40]). The intensities of the single PB steps observed with GFP$_{x1}$ were distributed in a skewed Gaussian-like manner (Figure 4-9, A, inset), and similar intensity-distributions were obtained for each PB step in the traces recorded for GFP$_{x2}$ and GFP$_{x4}$. The most likely PB step-size was similar
throughout, as indicated by the similar values of $\mu$ obtained from analyses in terms of the GEV distribution (Equation 4.4).

**Table 4-1. Summary of smPB analysis results of the GFP multiplexes**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Max Number of PB Steps, $n$</th>
<th>Probability of Detection, $p$</th>
<th>Average Number of PB Steps, $n_{ave}$</th>
<th>Most Likely Step Intensity, $\mu$ (kcps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP$_{x1}$</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>$1.24 \pm 0.05$</td>
</tr>
<tr>
<td>GFP$_{x2}$</td>
<td>2</td>
<td>$0.89 \pm 0.04$</td>
<td>1.8</td>
<td>$1.28 \pm 0.02$</td>
</tr>
<tr>
<td>GFP$_{x4}$</td>
<td>4</td>
<td>$0.71 \pm 0.02$</td>
<td>2.8</td>
<td>$1.13 \pm 0.03$</td>
</tr>
</tbody>
</table>

Agreement between the smPB histograms and the binomial distribution allows for the calculation of the probability $p$ that an individual fluorophore within the multiplex will be detected as a PB step. Depending on the scenarios, the probability $p$ can usually relate to factors such as the photophysics of excitation and emission, the photo-viability of the sample, or the threshold of detection of the microscope. To examine this relationship, GFP$_{x4}$ was excited at different intensities from 60 to 260 W/cm$^2$. At the lowest power, the smPB histogram was dominated by traces containing 1 and 2 steps, whereas at the highest power, 3- and 4-step traces were predominant (Figure 4-9 D). The fraction of 4-step PB traces increased from 2% at 60 W/cm$^2$ to about 50% at 260 W/cm$^2$ (Figure 4-10).

**Figure 4-10.** The probability of excitation and detection ($p$) and the percentage occurrence of 1-step traces (blue bars) and 4-step photobleaching traces (red bars) for GFP$_{x4}$ at different
excitation laser intensities. The green dots and accompanying error bars represent the values and uncertainties of the fitted probabilities that an individual fluorophore within the GFP×4 is detected as a PB step (i.e. \( p \) in Equation 4.3). The shaded band indicates the range of excitation intensity used in all subsequent experiments on the M2 receptor and Gi1, except when stated otherwise. The dashed line represents the best fit from an empirical analysis of \( p \) in terms of a single-exponential rise.

The sensitivity to the excitation power suggests that the parameter \( p \) is mostly determined by the chance of exciting a single fluorophore within the multiplex and detecting a smPB event within a noisy trace [41, 42]. There also may be a minor fraction of GFPs that cannot fluoresce owing to pre-bleaching, misfolding, or incomplete maturation of the fluorophore. Such dark elements may account for some particles with fewer than the expected number of PB steps and for the apparent upper limit of 80–90% on \( p \) at high laser-power (Figure 4-10). Considering the diminishing gain in simultaneously exciting more fluorophores versus the shortened duration of each PB step at high laser power, 180W/cm² was decided as the optimal excitation laser intensity for the subsequent M2 receptor and Gi1 protein samples (shaded band in Figure 4-10).

These observations indicate that a multiplexed fluorophore such as GFP×4 will yield a distribution of photobleaching steps in which the maximum number of steps is the same as the size of the oligomer. The measurements of GFP-tagged receptors and G proteins described below were taken under excitation conditions sufficient to excite multiple units of GFP within a complex while limiting their simultaneous or premature photobleaching and avoiding traces of short duration or low signal-to-noise ratios.

4.3.4 Photobleaching of the M2 receptor

M2 receptor fused at the N-terminus to GFP was purified and immobilized via a hexahistidyl tag on the fluorophore (His6-GFP-M2, Figure 4-3). The intensity traces of individual particles in TIRF images displayed up to 6 photobleaching steps (Figure 4-11), with ~80% of the particles showing more than two steps (Table S3). The distribution of the number of PB steps is consistent with the binomial distribution (Equation 4.3) with the max number of steps \( n \) taken as 6 (Figure 4-11, A). The fitted value of \( p = 0.594 \) from
the binomial distribution corresponds to an average of 3.6 PB steps per particle (i.e., $N_{\text{avg}} = np$) (Table 4-2).

![Figure 4-11.](image)

**Figure 4-11.** Distributions of the number of PB steps detected on the GFP-tagged M$_2$ receptor samples (His$_6$-GFP-M$_2$) without receptor ligand (A), or with agonist carbachol (B, dark blue bars), or with antagonist NMS (B, light blue bars). A maximum of 6 steps were observed, with an average number of 3.6 steps per molecule. The arrows in panel A identify the steps for which the distribution of step-wise intensities are analyzed in Figure 4-12.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Max Number of PB Steps, $n$</th>
<th>Probability of Detection, $p$</th>
<th>Average Number of PB Steps, $n_{\text{ave}}$</th>
<th>Most Likely Step Intensity, $\mu$ (kcps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>His$_6$-GFP-M$_2$</td>
<td>6</td>
<td>0.59 ± 0.02</td>
<td>3.6</td>
<td>1.76 ± 0.02</td>
</tr>
<tr>
<td>His$_6$-GFP-M$_2$ +10µM carbachol</td>
<td>6</td>
<td>0.55 ± 0.03</td>
<td>3.4</td>
<td>1.21 ± 0.02</td>
</tr>
<tr>
<td>His$_6$-GFP-M$_2$ +10µM NMS</td>
<td>6</td>
<td>0.56 ± 0.02</td>
<td>3.3</td>
<td>1.22 ± 0.03</td>
</tr>
</tbody>
</table>

Table 4-2. Summary of smPB analysis results of M$_2$ receptor samples.

The distribution of intensities for each PB step from 1 through 4 resembles the GEV distributions from controls of multiplexed GFP (Figure 4-9, A), suggesting that each of the first four PB steps in traces from the receptor corresponds to a single GFP. In contrast, the distributions of intensities for PB steps 5 and 6 showed more irregular distributions and could not be described by a single-component GEV distribution ($\chi^2 > 5$). Such anomalous behavior may arise from fluorescent impurities, which are known to bleach.
faster than the photostable fluorophores used in single-molecule studies [43]. They therefore would appear as higher-indexed events in the intensity-time trace, where the photobleaching steps are numbered from latest to earliest (Figure 4-5 B). Although the origin of steps 5 and 6 is unclear, the patterns displayed by steps 1–4 are indicative of particles with four fluorophores, such as a tetramer.

![Graphs showing step intensities](Figure 4-12)

**Figure 4-12.** The distributions of step intensities for the 1st, 4th, 5th, and 6th steps displayed on the photobleaching trajectories of immobilized M2 receptors in Figure 4-11, A. PB steps 1 to 4 yielded intensity distributions that can be described by the GEV model, whereas the 5th and 6th step displayed more irregularity and cannot be fitted by the GEV model.

Muscarinic ligands had little or no effect on the distribution of PB steps (cf. Figure 4-11). The value of $N_{avg}$ calculated for the vacant receptor from the fitted estimate of $p$ is 3.6; the corresponding values for the receptor plus the agonist carbachol and the inverse agonist NMS are 3.43 and 3.27, respectively, at a saturating concentration of each ligand (Table 4-2). Similarly, the distributions of PB intensities obtained with liganded receptors resembled those of the vacant receptor, including the anomalous behavior of steps 5 and 6. The distribution shown in Figure 4-11 (A) was obtained in the absence of a reducing agent, but essentially the same pattern was obtained in the presence of 10 mM DTT. This
latter observation and the results from multiplexed controls suggest that the multimeric particles identified in the photobleaching patterns of the M₂ receptor were not a product of disulfide-mediated aggregation or clusters of protomers within the same diffraction-limited spot; rather, they were extracted, purified, and immobilized as non-covalent clusters.

4.3.5 Photobleaching of the Gᵢ protein

Holo-G proteins containing GFP-Gα₁ₒ were purified and immobilized via a hexahistidyl tag at the N-terminus of Gγ₂ (GFP-Gα₁ₒβ₁γ₂-His₆) (Figure 4-3), thereby ensuring that photobleaching measurements were done on heterotrimers. DTT was present throughout at a concentration of 10 mM. The intensity traces displayed up to 6 smPB steps, with 92% of the particles showing more than two steps (Figure 4-13, A). The smPB histogram was described by the binomial distribution (Equation 4.3) with n taken as 6, and the fitted value of p (69.4%) corresponds to an average of 4.2 PB steps per particle (N_avg) (Table 4-3).

![Figure 4-13](image). Distributions of the number of PB steps detected on the GFP-tagged holo-Gᵢ protein samples (GFP-Gα₁ₒβ₁γ₂-His₆) without GTP (A), or with GTP (B, light brown bars), or with a GTP structural mimic, GDP+AlF₄⁻ (B, dark brown bars). A maximum of 6 steps were observed, with an average number of 4.2 steps per molecule. The arrows in panel A identify the steps for which the distribution of step-wise intensities are analyzed in Figure 4-14.

The data illustrated in Figure 4-13 are in good agreement with the binomial theorem when n = 6 (Equation 4.3), and the fit is compromised at higher or lower values of n (see
also the SI Section S4, Table S5 of the manuscript [40]). In the case of an octamer \((n = 8)\), for example, particles with 7 photobleaching steps are predicted in numbers that are above the threshold of detection, but were not observed. Endogenous G proteins are a form of dark protomers, and their stochastic incorporation into a multimeric complex of defined size would reduce the number of GFP-tagged protomers in a predictable manner; in an oligomer of size \(n\), such a dilution would be accompanied by countervailing increase in the probability of exciting and detecting a fluorophore, \(p\).

**Figure 4-14.** The distributions of step intensities for the 1st, 4th, 5th, and 6th steps displayed on the photobleaching trajectories of immobilized \(G_i\) proteins in Figure 4-13, A. The step intensities from all 6 steps can be described well by the GEV model.

All the 6 PB steps shown in Figure 4-13 gave distributions of intensities (Figure 4-14) that were skewed like those of multiplexed GFP, with consistent average intensity \(I_{\text{avg}} \approx 1,900 \text{ cps}\). Each of the 6 steps therefore appears to represent a single GFP fluorophore, in contrast to the anomalous patterns obtained for steps 5 and 6 in the case of the receptor (Figure 4-12). The consistency among all 6 PB steps suggests that \(G_{i1}\) is a hexamer in the absence of the receptor.

The addition of GTP\(\gamma\)S or the sequential addition of GDP and AlF\(4^-\) caused a small leftward shift in the smPB distributions (Figure 4-13, B) (AlF\(4^-\) here serves as a mimic of
a phosphate PO₄⁻. When bound to GDP, the two molecules yield a structural mimic of GTP [44, 45]). The maximum number of PB steps remained unchanged at 6. In terms of the binomial distribution (Equation 4.3, \( n = 6 \)), the value of \( N_{\text{avg}} \) decreased marginally from 4.2 in the absence of ligand to 3.8 with GTP\( \gamma \)S or to 3.9 with GDP followed by AlF₄⁻ (Table 4-3). The intensity distributions for all 6 PB steps were similar to those in the absence of ligand. Because the level of occupancy by GTP\( \gamma \)S was about 90% (\( \log K = -6.01 \)), the 5- and 6-step traces likely arise from fully occupied \( G_{i1} \) rather than from a subpopulation of vacant \( G \) proteins. Guanylyl nucleotides therefore do not appear to affect the oligomeric state of heterotrimeric \( G_{i1} \).

### Table 4-3. Summary of smPB analysis results of \( G_i \) protein samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Max Number of PB Steps, ( n )</th>
<th>Probability of Detection, ( p )</th>
<th>Average Number of PB Steps, ( n_{\text{ave}} )</th>
<th>Most Likely Step Intensity, ( \mu ) (kcps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-( \alpha_i )β₁- Hi₅₋γ₂</td>
<td>6</td>
<td>0.69 ± 0.02</td>
<td>4.2</td>
<td>1.91 ± 0.02</td>
</tr>
<tr>
<td>GFP-( \alpha_i )β₁- Hi₅₋γ₂ + GDP + AlF₄⁻</td>
<td>6</td>
<td>0.65 ± 0.04</td>
<td>3.9</td>
<td>1.74 ± 0.02</td>
</tr>
<tr>
<td>GFP-( \alpha_i )β₁- Hi₅₋γ₂ + GTP( \gamma )S</td>
<td>6</td>
<td>0.64 ± 0.03</td>
<td>3.8</td>
<td>1.81 ± 0.03</td>
</tr>
</tbody>
</table>

The amount of endogenous \( G \) protein was quantified by radio ligand binding. \( Sf9 \) cells express substrates for pertussis toxin [46], and in some cases their levels may be similar to those of exogenous \( \alpha_i \). The specific binding of \[^{35}\text{S}]\text{GTP}\( \gamma \)S in extracts from uninfected \( Sf9 \) cells was 45–65% of that in extracts of cells expressing His₆-tagged \( \alpha_{i1} \), measured at radioligand concentrations of 1.0 and 0.1 \( \mu \)M and expressed relative to total protein. It nevertheless appears that few if any endogenous \( G \) proteins were incorporated into oligomers of GFP-\( G_{i1} \): as argued below, such an incorporation would affect the fitted probability of detecting a single fluorophore but not the PB-based estimate of oligomeric size.

The Influence of endogenous \( G \) proteins on the smPB measurements (dark protomers) was evaluated by fitting the distribution of PB steps to Equation 4.2. In the case of GFP-
G\textsubscript{i1} and \( n = 6 \), the probability of detecting a fluorophore would increase from 0.7 in the absence of dark protomers to 0.78, 0.88 and 1.0 with dark protomers at mole fractions of 0.1, 0.2, and 0.3, respectively. Fitted values of \( p \) were \( \sim 0.8 \) or less for GFP\textsubscript{x4} and \( \sim 0.6 \) for oligomers of the M\textsubscript{2} receptor (Table 4-1 and Table 4-2), thus suggesting that the mole fraction of endogenous G proteins in oligomers of GFP-G\textsubscript{i1} cannot exceed 0.1–0.2. That limit would be lower if protomers of G\textsubscript{i1} were favored over the endogenous G proteins of Sf\textsubscript{9} cells during assembly of the oligomer. Such a preference is consistent with evidence that the G proteins of Sf\textsubscript{9} cells do not couple to M\textsubscript{2} muscarinic receptors [46]. Finally, extracts of Sf\textsubscript{9} cells were immunonegative when probed with antibodies to mammalian G\textsubscript{\alpha\textsubscript{i}} [1-3] or G\textsubscript{\alpha\textsubscript{o}} [46].

4.4 Discussion

G protein-coupled receptors are known to form oligomers in live cells and other preparations [17, 20, 47], and those oligomers have been studied at some length for their prevalence, stability, and biological role [48]. In contrast, oligomers of G proteins have received relatively little attention. Aggregates of G\textsubscript{s}, G\textsubscript{i}, G\textsubscript{o}, and G\textsubscript{q} were detected early on in detergent-solubilized extracts from rat brain, and their disaggregation was implicated in signaling [49]. Also, the allosteric interaction between agonists acting at the receptor and guanylyl nucleotides acting at the G protein has prompted the suggestion that oligomers of GPCRs imply oligomers of G proteins [50]. Coupling of two such oligomeric partners would be expected to yield a ligand-sensitive complex of receptors and G proteins.

Oligomers of the M\textsubscript{2} receptor alone have been confirmed by our smPB analysis, in which immobilized particles of the purified GFP-tagged receptor displayed up to 6 photobleaching steps. The distributions of intensities from four of those steps resembled the distributions from single fluorophores in multiplexed GFP controls, suggesting that the receptor was purified as a tetramer. The oligomeric state of the receptor was essentially unaffected by muscarinic ligands, in that the average number of PB steps was virtually identical in the absence of any ligands and in the presence of saturating levels of the agonist carbachol or the antagonist NMS. These observations agree with previous results based on mechanistic modeling [8, 13], electrophoretic mobility after cross-
linking [13], and FRET between fluorophore-tagged protomers in live cells [20], all of which have suggested that the M₂ muscarinic receptor exists at least in part as a tetramer.

Several pieces of experimental evidence indicate that Gα₁₁ and holo-Gi₁ also exist as oligomers, both in live CHO cells and after purification from Sf9 membranes. Multimeric forms of Gi₁ in CHO cells were detected by dcFCS (for details, see also Chapter 3.3.5.3) and, independently, by FRET between α-subunits tagged with GFP or mCherry. The degree of cross-correlation between the two spectral channels in dcFCS indicated that at least 50% of the diffusing particles contained both fluorophores, which is an unambiguous signature of proteins that co-diffuse as oligomers (Figure 4-6).

The FRET efficiency estimated with holo-Gi₁ increased with the ratio of acceptor to donor in a saturable manner, leveling off at $E_{\text{max}} = 50–75\%$ (Fig. 2C in the published manuscript [40]). That dependence was invariant over a 64-fold range of total expressed GFP- and mCherry-tagged Gα₁₁; also, the levels of expression at the lower end of that range were similar to those in cells that displayed a significant degree of cross-correlation in dcFCS (Figure 4-6 and Chapter 3.3.5.1). Model-based estimates of the pair-wise FRET efficiency between a single donor and a single acceptor suggest that oligomers of Gi₁ detected by FRET comprised at least four heterotrimeric G proteins (Fig. 2C in the published manuscript [40]). The possibility that the interactions detected by dcFCS and FRET contain a stochastic component was ruled out in parallel studies with a non-interacting control (Figure 4-8).

In agreement with the FRET-based estimate of the oligomeric size in CHO cells, the single-molecule photobleaching data point to hexamers of GFP-tagged Gα₁₁ purified from Sf9 cells with or without or Gβ₁γ₂. The average number of PB steps per particle was the same for Gα₁₁ ($N_{\text{avg}} = 3.8$ steps) and holo-Gi₁ ($N_{\text{avg}} = 3.9$ steps), suggesting that the oligomer of holo-Gi₁ is stabilized primarily by interactions between the α-subunits. The reducing environment experienced by G proteins in live cells [51] was maintained during extraction and purification by the inclusion of DTT at a concentration of 10 mM. Reactive cysteine residues therefore are unlikely to account for the stability of oligomers identified by stepwise photobleaching.
In single-particle studies of fluorescently labelled proteins, oligomeric size is inferred from the number of photobleaching steps and their intensities. To calibrate this relationship, we examined the photobleaching patterns of purified controls comprising single and multiplexed units of GFP (Figure 4-1, A). Each control was purified from *E. coli* and immobilized in the same manner as the GFP-tagged M₂ receptor and Gᵢ₁ protein. The complement of fluorophores was confirmed by its electrophoretic mobility (Figure 4-1, B). Almost all spots of unitary GFP showed a single PB step (~97%, Figure 4-9, A), and the number of steps observed with duplex and quadruplex GFP never exceeded 2 or 4, respectively (Figure 4-9, B and C). It follows that the intensity within a diffraction-limited spot derived from one and only one molecule of the control or, by extension, from one complex of GFP-tagged receptor or Gᵢ₁.

When single particles of labeled GPCRs were tracked previously in live cells [23, 52], the fluorescence intensities gave skewed Gaussian-like distributions similar to those described here (e.g., Figure 4-12 and Figure 4-14). It was assumed in the analyses of those distributions that the signal detected from a single fluorophore under a TIRF microscope is normally distributed, leading to the conclusion that the receptors were predominantly monomeric with a minor contingent of dimers. A different picture emerges from our measurements of single and multiplexed GFP, which show that a skewed distribution of intensities is intrinsic to a single fluorophore (Figure 4-9 A, inset). Such asymmetry could arise from an inhomogeneous illumination field in the objective-based TIRF microscope and from the readout noise associated with weak signals in the electron-multiplying CCD camera.

The distribution of the number downward intensity steps occurring in single-particle PB traces typically is attributed to a mixture of species that differ in oligomeric size or, more specifically, in the number of fluorophores. An alternative explanation emerges from our experiments with purified multiplexed controls, where the distribution of intensities obtained with GFPₓ₄ was shifted to the right or the left by changes in the laser excitation intensity. Such behavior indicates that the shape of the distribution is not a manifestation of heterogeneity within the sample; rather, it is a measure of the number of fluorophores that are simultaneously excited and detected within the multiplex or oligomer. One of the
main results of this study is that the distribution of the number of PB steps is in fact a binomial distribution defined by the total number of fluorophores (i.e., tagged monomers) within the oligomer and the independent probability of exciting and detecting a single fluorophore.

4.5 Conclusions

We have determined the supramolecular organization of the M$_2$ muscarinic receptor and holo-G$_{i1}$ by single-molecule photobleaching of GFP fused to immobilized receptors or G$_{a1}$-subunits. The receptor and the G protein were purified from Sf9 cells and the oligomeric status of each was inferred from statistical analyses of the number of photobleaching steps. The M$_2$ receptor was purified as a tetramer, and the oligomeric state was unaffected by muscarinic ligands; G$_{i1}$ alone was purified as a hexamer that was unaffected by GTP$_{\gamma}$S. Oligomers of G$_{i1}$ larger than dimers were identified in live CHO cells by dcFCS and FRET, indicating that such structures are biologically relevant.

Additional smPB data acquired in the Gradinaru lab has identified a purified complex of the agonist-bound receptor and nucleotide-free G protein as a hetero-octamer comprising a tetramer of the M$_2$ receptor and a tetramer of heterotrimeric G$_{i1}$ (Fig. 6 in the published manuscript [40]). Activation of the complex by GTP$_{\gamma}$S plus the agonist carbachol was accompanied by a reduction in the oligomeric size of G$_{i1}$ from a tetramer to dimers and perhaps to monomers, while the receptor remained a tetramer. The basal and active states of G$_{i1}$ therefore appear to differ in oligomeric size.

Our results provide direct evidence for oligomers that have been inferred previously from mechanistic analyses of the binding properties of the M$_2$ muscarinic receptor and its attendant G proteins [8, 9, 50]. Disassembly of the oligomeric G protein within such a hetero-oligomeric signalling complex may allow the receptor to engage other effectors, leading to activation of different pathways and multiple signaling outcomes.
4.6 References


5 Single Particle Tracking of Receptors and G Proteins in Live Cells

5.1 Introduction

With the developments of fluorescence microscopy and computerized image processing, single particle tracking (SPT) is becoming an increasingly powerful tool for studying biomolecules in live cells. In particular, SPT on Total Internal Reflection Fluorescence (TIRF) Microscope excels at observing membrane proteins, enabling simultaneous analysis of all the fluorescently tagged membrane proteins at high signal-to-noise ratio. In terms of temporal resolution, the TIRF-based SPT technique is an extension to the confocal spectroscopy, covering slow processes within 1ms~10s temporal scale and 0.1um~10um spatial scale.

In the field of GPCR research, SPT has been used on a wide collection of receptors to investigate the distribution, motion, and signaling dynamics in live cells under physiologically relevant conditions [1-4]. In this project, we focused on answering the questions about the oligomeric sizes of the M2 muscarinic receptors and Gi proteins, and about the coupling interaction between the M2 receptor and Gi protein in response to the receptor ligands.

5.2 Material and Methods

5.2.1 Live-cell measurements on the total internal reflection fluorescence microscope

Detailed cell culture and transfection protocol can be found in Chapter 3.2.1.2. In brief, CHO cells were grown in MatTek glass bottom dishes. Prior to the experiments, the cell culture medium was changed to colorless phenol-free minimal essential medium, with supplement of 25mM HEPES to maintain optimal pH under regular atmospheric conditions. The glass bottom dishes were kept at constant 37°C inside an on-stage heating chamber during the course of the experiments. The cells were found viable for at least one hour under these experimental conditions, sufficient for the SPT measurements on membrane proteins.
All of the experiments were conducted on the TIRF Microscope in the Gradinaru Lab. The details of the equipment can be found in Chapter 2.3.1. In brief, the blue 473nm laser (Cobolt Blue, Colbolt AB) and the green 532nm laser (LRS-0532, Laser Glow) were used to excite the GFP- and mCh-labeled molecules expressed in live CHO cells. The fluorescence images of the fluorescent particles in cells were collected by the EMCCD camera (Andor iXon DU-897BV), and then stored as image sequence stacks (.tiff files). The images stacks were then analyzed by a specialized SPT software to retrieve the diffusion information of the fluorescent particles.

5.2.2 Single particle tracking algorithms

There are dozens of SPT software packages available online with various strengths and weaknesses [5]. Several recent studies have been published, comparing the performance between the most commonly used tracking software packages. Using our live-cell data, we evaluated three different SPT software packages (see Table 5-1 and Figure 5-1). Judging by tracking precision, expandability, and computational cost of the packages, we opted for the feature point tracking algorithm developed by K. Jaqaman [6] and G. Danuser et. al. [7]. The package has a user-friendly GUI with access to the advanced options and is available as an ImageJ plugin called TrackMate.

Almost all particle tracking algorithms involve two distinct steps: the particle detection step and the trajectory linking step. For the feature point tracking algorithm, the particle detection step is carried out by an iterative intensity-weighted centroid calculation method; subsequently, the trajectory linking step employs a greedy hill-climbing optimization with a user-tunable cost function for particle features.

SPT, as a NP hard problem (a problem that is as hard as nondeterministic polynomial time), faces various challenges [5]. The most notable challenges for tracking membrane proteins include particle motion heterogeneity, temporary particle disappearance, particle merging and splitting, fluorophore blinking, and autofluorescence background. In order to find the best performing software package for our equipment and samples, we tested three packages on a few TIRF movies of GFP- or mCherry labeled M2 receptors or Gi proteins with an average signal-noise ratio of ~4, which is relatively low for typical
single-molecule tracking studies. The feature point tracking mentioned above was chosen for its reliability and tunability at low cost of computation power.

**Table 5-1. Features of the different SPT algorithms that were tested in this study**

<table>
<thead>
<tr>
<th></th>
<th>Feature Point Tracking (<em>TrackMate</em>)</th>
<th>Multiple Hypothesis Tracking (<em>Icy</em>)</th>
<th>Nearest Neighbor Tracking (<em>Braeckmans Group</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle detection algorithm</td>
<td>Laplacian of Gaussian</td>
<td>Maxima detection + Thresholding</td>
<td>Global thresholding of size &amp; brightness</td>
</tr>
<tr>
<td>Trajectory linking algorithm</td>
<td>Multi-frame linear assignment problem considering particle brightness and shape</td>
<td>Bayesian whole-history optimization according to motion model</td>
<td>Nearest neighbor search allowing for reconnection of missing steps</td>
</tr>
<tr>
<td>Pros</td>
<td>Fast; tunable cost for imperfect linking</td>
<td>Most accurate*; deals with merging/splitting</td>
<td>Simple; fast; source code accessible</td>
</tr>
<tr>
<td>Cons</td>
<td>Less accurate* than <em>Icy</em></td>
<td>High computational cost</td>
<td>More frequent fragmentation of trajectories.</td>
</tr>
</tbody>
</table>

*: The tracking accuracy of *TrackMate* and *Icy* was quantitatively evaluated by Chenouard *et. al.* [5] using simulated data with pre-defined diffusion properties.

Due to the unavailability of simulated movies of particles in live cells that exactly match our experimental conditions, we compared the performance of the tracking algorithms using a few typical movies gathered with the GFP- or mCherry-tagged molecules in live CHO cells. Two reporting factors were taken into consideration for quantitative evaluation of the performance, namely (1) the total number of trajectories detected, and (2) the average duration of trajectories.
Figure 5-1. A comparison between the TrackMate program and the tracking software developed by the Braeckmans Group at the Ghent University, Belgium [8, 9]. GFP-tagged M₂ receptors in three cells (Sample ID 1, 2, 3) were tracked with both programs. The total number of trajectories detected by each program (left panel) and the average number of frames per trajectory (right panel) were used as benchmarks to evaluate the performance of the two SPT algorithms.

Generally speaking, the tracking program that yields a lower number of trajectories and a higher average trajectory duration is preferred because it indicates less frequent fragmentation of single-particle trajectories. Thus, we chose TrackMate based on its better benchmarks (Figure 5-1). However, it should be noted that these two benchmarks are not exhaustive, as false positive identifications of particles and incorrectly joining unrelated fragmented trajectories could artificially decrease the number of trajectories and/or increase the average duration of the trajectory. Better, more rigorous tests are currently underway in the Gradinaru Lab based on simulated data and on data obtained from control measurements of dyes in supported lipid bilayers.

5.2.3 Analysis methods of the tracked trajectories

Ignoring the merging/splitting events, the tracked trajectories of particle positions can be described as a collection of arrays, each array composed of three columns, the x position, the y position, and the time point t, i.e. \((x, y, t)\). Instantaneous speed/angle analysis could be performed directly on each of the arrays. The trajectories could also be overlaid directly at a common starting position to show the possible inhomogeneity in the
directions of the motion of the particles. Single-molecule trajectories were typically analyzed using a mean squared displacement calculation.

**MSD calculation**

The most informative analysis of SPT trajectories is the calculation and fitting of mean squared displacement (MSD). The MSD function is defined as

\[ MSD(\tau) = \langle \Delta x^2(\tau) + \Delta y^2(\tau) \rangle \]  

Equation 5.1

where \( \tau \) is the lag time between two positions of the particle and the average is taken either along a single trajectory or across a population of particles.

**Individually time-averaged MSD (TA-MSD)**

As a benefit of the single molecule resolution of the TIRF microscope, the MSD function for each particle can be calculated separately as:

\[ MSD(\tau) = \sum_{t=0}^{T-\tau} \left[ (x(t+\tau) - x(t))^2 + (y(t+\tau) - y(t))^2 \right] \]  

Equation 5.2

where \( T \) is the total number of frames in the trajectory. The TA-MSD describes the diffusion behavior of a single particle.

**Ensemble-averaged MSD**

In addition to the individually time-averaged MSD, one can also compute the ensemble time-averaged MSD considering all tracked trajectories.

\[ MSD_{population}(\tau) = \sum_{i=1}^{N} \sum_{t=0}^{T-\tau} \left[ (x_i(t+\tau) - x_i(t))^2 + (y_i(t+\tau) - y_i(t))^2 \right] \]  

Equation 5.3

where \( N \) is the total number of trajectories that are included in the calculation. The ensemble time-averaged MSD provides an overview of all the particles in the same group, enabling the comparison of different motion behaviors between multiple cells, varying conditions, and the types of molecules tracked.
MSD Fitting models

According to the random walk theory, a free Brownian diffusion in two dimensions exhibits a MSD function that is linearly proportional to the lag time $\tau$:

$$MSD(\tau) = 4D\tau$$  \hspace{1cm} \text{Equation 5.4}

In practice, the trajectories of the tracked particles often showed a variety of diffusion modes. A non-linear anomalous diffusion model was introduced for the simultaneous analysis of all the TA-MSD functions [10, 11]:

$$MSD_{\alpha}(\tau) = 4D_{\alpha}\tau^{\alpha} + \sigma^2$$  \hspace{1cm} \text{Equation 5.5}

where $\alpha$ is the anomalous diffusion factor and $\sigma$ is the precision of localization (PoL). The anomalous factor $\alpha$ reflects the non-Brownian behavior of the diffusing particles, and was employed for categorizing the motion behaviors, i.e., confined, free, or transported (see below). The PoL parameter $\sigma$ is the time-independent displacement of the particles, and describes the measurement uncertainty about the position of the particles at any given time (subject to the influence of signal-noise-ratio). Any particle with a total displacement less than the $\sigma$ value can be treated as immobile. The PoL is estimated and discussed in details in Section 5.3.1., by taking into account the imaging resolution of the TIRF microscope and the tracking algorithm of the TrackMate method.

The anomalous factor $\alpha$ serves as a criterion for categorizing particles into physically different diffusion regimes. For the scope of this study, the specific diffusion regimes that were discriminated by $\alpha$ included: free normal diffusion ($0.8 < \alpha < 1.2$), sub-diffusion due to confinement ($\alpha < 0.8$), and super-diffusion due to active transport ($\alpha > 1.2$). Different cells imaged might have had different fractions of particles in each diffusion regime, and these fractions might vary depending on the presence of various receptor ligands. Therefore, we analyzed each diffusion regime separately.

The MSD functions of the particles showing sub-diffusion were re-fitted with the confined diffusion model [1, 12, 13]:

143
The MSD functions of the particles showing super-diffusion were further analyzed to calculate the root-mean-square speed (RMS speed, $v_{\text{RMS}}$), in order to obtain the distribution of the average speeds of the active transport of receptors in the cell. More details about the RMS speed analysis are presented in Section 5.3.8.

### 5.2.4 Single-molecule photobleaching in fixed cells

Due to the excessive fluorescence fluctuations in the SPT trajectories, it was difficult to analyze the intensity traces of the mobile single particles in live cells using our current software [15]. A method based on Bayesian analysis of noisy traces rather than discernible discrete steps had been developed by our collaborator, Prof Andrew Rutenberg, and will be applied in the future for this type of data (manuscript to be submitted to Biophysical Journal sept. 2017).

In order to estimate the number of fluorophores in the tracked particles, we performed single-molecule photobleaching (smPB) experiments on immobilized membrane proteins in fixed CHO cells. Prior to the measurements, CHO cells expressing the fluorescently labeled proteins were incubated in 4% formaldehyde at 4°C for 30min. Formaldehyde fixated the proteins inside the cell through cross-linking the lysine residues on the proteins with nearby nitrogen atoms in other protein residues or DNA.

smPB experiments have strict requirements on the expression level of the fluorescently tagged molecules. The density of the molecules must be low so that the probability of aggregation would be low, and that individual particles can be distinguishable from one another given the optical resolution of the instrument (~0.5 μm). While the density of
molecule is kept below the threshold for aggregation, higher densities of molecules are preferred to ensure efficient and fast data acquisition. The density of molecules is governed by the expression level in cell, which is further affected by the transfection quantity of DNA plasmid, the incubation time after transfection, and the nature of the reconstituted protein, *i.e.*, more complex multi subunit proteins will generally exhibit lower expression levels.

For each protein sample measured in fixed cells, a series of cells with various transfection conditions were tested and the optimal conditions were determined through visual comparison. GFP-fused M₂ receptors and Gᵢ proteins were expressed in separate CHO cell cultures and then imaged with the TIRF microscope. Within the scope of the current study, single-color measurements were sufficient for quantification of the oligomeric sizes of the M₂ receptor and the Gᵢ protein, respectively. Further dual-color experiments will be aimed at measuring the oligomeric sizes of the two proteins when they form an active signaling complex together.

### 5.3 Results and Discussion

#### 5.3.1 Signal/noise ratio and precision of localization

It has been previously reported that the signal-to-noise ratio (SNR) is one of the key factors affecting the performance of the single particle tracking analysis [16, 17]. The definition of the SNR could take on various forms, yielding different values for the same set of data. In practice, it is usually sufficient to compute the SNR using a particular definition and then compare the influence of different SNR values on the tracking performance in a relative manner.

In our application, we chose to use the SNR definition as specified by the *TrackMate* built-in function:

\[
SNR = \frac{I_{in} - I_{out}}{\sigma_{in}}
\]

Equation 5.7
where $I_{in}$ and $I_{out}$ represent the mean intensity inside and outside of a detected particle, respectively, and $\sigma_{in}$ represents the standard deviation of the intensity fluctuations within the detected particle. This definition was used for the calculation of SNR values for all subsequent analysis.

In order to determine the minimally acceptable SNR for reliable SPT analysis, we evaluated the influence of SNR on the tracking performance of immobile fluorescent beads. 100-nm fluorescent beads (TetraSpeck Microspheres, ThermoFisher, T7279) were imaged on our TIRF microscope. The blue 473-nm laser was attenuated to provide lower excitation powers and to simulate the levels of SNR that are typically observed on fluorescent particles in live cells. The exposure time of the camera was set at 20 ms per frame, with 500 frames per movie, resulting in a total duration of measurement of 10 seconds. Movies of the fluorescent beads inside the same region of interest (ROI) were collected using a range of excitation powers, from 5.2 to 31.2 W/cm$^2$ measured at the sample. The ROI was chosen such that there were no other beads located within a 5-pixel radius around any given bead. The short duration of the imaging and the photostability of the fluorescent beads ensured that the intrinsic brightness of each bead did not change over the course of the measurement.

The data were analyzed with the TrackMate tracking program. Although there was no apparent displacement of the calibration beads during the course of the measurements, the program experienced more difficulties in detecting and tracking the beads under lower excitation powers (see Figure 5-2). As indicated by the arrows in Figure 5-2, under these conditions, some particles were missed by the identification algorithm, and there were some false positive detections at locations where there were no beads present.
Figure 5.100-nm fluorescent beads in the same ROI under three different excitation/SNR conditions. The raw images of the ROI are shown in panels A–C, the particles identified by the TrackMate program are shown with purple circles in panels D–F. The excitation laser intensity was 5.2 W/cm\(^2\) for panels A and D, 7.8 W/cm\(^2\) for panels B and E, and 31.2 W/cm\(^2\) for panels C and F. The particles identified by the tracking program are shown in magenta circles in panels D–F. Missed detections and false positive detections in panels D and E compared to panel F are marked by yellow arrows.

In the linking step of TrackMate, the detected positions of the beads were linked to create motion trajectories by allowing up to 2 pixels of displacement (~0.35 μm) per frame and 5 frames (0.1 sec) of detection gap. The linked motion trajectories composed of more than 25 frames were exported to MATLAB and analyzed for time-averaged mean squared displacement (TA-MSD) according to the method outlined in Section 5.2.3. The precision of localization, \(\sigma\), and the apparent free diffusion coefficient, \(D\), under each excitation power were extracted from the ensemble-averaged MSD function by fitting to Equation 5.5, with the anomalous factor \(\alpha\) fixed to 1.
In addition, for each tracked particle, the SNR was calculated using Equation 5.7. The results are summarized in Table 5-2 for different excitation intensities (SNRs).

**Table 5-2. Summary of the MSD fitting results for surface-immobilized fluorescent beads.** *

<table>
<thead>
<tr>
<th>Excitation Intensity (W/cm²)</th>
<th>Minimal SNR</th>
<th>Average SNR</th>
<th>Fitted Diffusion Coefficient (D) ((\mu m^2/s))</th>
<th>Fitted Precision of Localization (\sigma) ((\mu m))</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2</td>
<td>3.74</td>
<td>4.95</td>
<td>6.24\times10^{-4}</td>
<td>0.149</td>
</tr>
<tr>
<td>7.8</td>
<td>3.72</td>
<td>5.44</td>
<td>5.5\times10^{-4}</td>
<td>0.135</td>
</tr>
<tr>
<td>10.4</td>
<td>3.74</td>
<td>5.87</td>
<td>5.08\times10^{-4}</td>
<td>0.121</td>
</tr>
<tr>
<td>15.6</td>
<td>3.83</td>
<td>7.11</td>
<td>1.65\times10^{-4}</td>
<td>0.101</td>
</tr>
<tr>
<td>20.8</td>
<td>5.35</td>
<td>8.98</td>
<td>1.24\times10^{-5}</td>
<td>0.078</td>
</tr>
<tr>
<td>31.2</td>
<td>5.90</td>
<td>11.0</td>
<td>1.96\times10^{-5}</td>
<td>0.066</td>
</tr>
</tbody>
</table>

* The laser excitation power was measured after the objective, at the sample position. SNR values were calculated for each individually tracked bead; the average SNR is an ensemble average of the values collected from all of the beads.

It can be noticed from Table 5-2 that the minimal SNR of all detected beads was ~3.7. By comparison, the lower limit of SNR for reliable SPT was estimated to be around 4 by several previous studies [5, 16, 17]. A qualification criterion of SNR = 5 was therefore introduced for screening the SPT trajectories of the fluorescently labeled proteins in cells. As a consequence, any particle with SNR lower than 5 was automatically excluded from the diffusion analysis due to the impaired reliability of the program to correctly and reliably identify it.

Ideally, the calibration beads should appear to be completely immobile, showing no diffusion motion at all. However, due to the uncertainty in determining the positions of the particles, the values of the fitted diffusion coefficient \(D\) were non-zero. Nevertheless, the \(D\) values showed a decreasing trend with increasing SNR values. Based on this calibration data, two filters were applied to the motion analysis of the fluorescently labeled proteins diffusing in live cells (see below). After imposing that SNR > 5 (see above), a second criterion of \(D > 1 \times 10^{-3} \mu m^2/s\) was introduced to discard immobile particles.
These two criteria proved to be sufficient for rejecting most of the dim, noisy, or immobile particles. However, in some rare cases, the anomalous fitting model (Equation 5.5) yielded anomalous factors $\alpha$ close to 0 and diffusion coefficients large enough to pass the immobile limit. Then the PoL $\sigma$ values could be employed to further filter out the immobile particles. As shown in Table 5-2, $\sigma$ values decreased with increasing SNR, as more photons were available for the program to accurately determine the center of the particle. Accordingly, a third filtering criterion was introduced to discard any particle that showed a maximal displacement that was less than 0.2 μm at any given lag time $\tau$, which corresponded to ~1.2 pixels in the TIRF setup.

Low SNR not only increases the uncertainty in the determination of the positions of particles, but also causes missed identifications and segmented SPT trajectories. Inside the ROI that was analyzed in Figure 5-2 and Table 5-2, there was a total of 29 beads that were visible on the movie of the highest SNR (with 31.2 W/cm$^2$ excitation laser intensity), each of which were successfully detected throughout the 500 frames of the entire measurement. However, as is summarized in Figure 5-3, the fragmentation of the tracked trajectories became more frequent with the lower SNR values.

![Figure 5-3](image.png)

**Figure 5-3.** SNR and the tracking fragmentation of single-particle trajectories. A total of 29 beads were present in the ROI and the total number of acquisition frames in the movie was 500. Ideally, there should be 29 identified trajectories, with 500 frames per trajectory. When the trajectories were fragmented due to false negative or false positive detection, the total number of identified
trajectories would increase, while the average duration of the trajectories (i.e. number of frames that each trajectory lasted) would decrease.

As shown in Figure 5-3, at approximately SNR = 7, there were ~50% of the trajectories appearing as fragmented, leaving ~30% less frames per trajectory. Hence, we propose that an SNR of at least 7 is desirable for analyzing particles through extended periods of time. Unfortunately, such a high SNR is difficult to achieve for fluorescently labeled membrane proteins in live cells. Consequently, most of the work presented in this thesis was limited to analyzing fragmented SPT trajectories. The conclusions are therefore valid only with the spatial and temporal constraints determined by lower SNR values.

5.3.2 Optimization of exposure frame rate

In Section 5.3.1, the SNRs were kept low partly by using a very short exposure time per frame. Increased exposure time would allow more photons to be accumulated from the fluorescent molecules, thus increasing SNR. However, due to potential movement of the particles within the exposure time, a long exposure time could also introduce uncertainty in the positions of the detected particles. In order to find the optimal exposure time per frame, we quantitatively examined the influence of exposure time on the performance of tracking using the bead (100-nm TetraSpeck microspheres) sample.

A series of movies of the same ROI were recorded under different exposure times. The excitation power was adjusted so that the product of excitation power and exposure time remained constant, thus keeping the SNR approximately constant. For a fixed total of 500 frames, the total duration of the measurements therefore varied across the range of 12.5–200 sec. The SNR values were calculated with Equation 5.7. The MSD of the beads was modeled with Equation 5.5 assuming an anomalous factor $\alpha = 1$. The apparent diffusion coefficient $D$ and the precision of localization $\sigma$, along with the average number of frames per tracked trajectory are summarized in Table 5-3 below.

The results showed that, with SNR remaining approximately the same, the PoL was not significantly affected by nearly tenfold of changes in the frame rate. The exception was the measurement with the longest exposure time, 0.4 sec, of which the tracking quality appeared to be the worst, possibly because of the influence from the overall mechanical
stability of the TIRF microscope. Based on this test, all subsequent measurements were performed with 0.05~0.1 sec frame exposure time, in order to minimize the localization uncertainty introduced by the mechanical stability of the setup.

Table 5-3. The effect of frame exposure time on the performance of single-particle tracking.

<table>
<thead>
<tr>
<th>Excitation Intensity (W/cm²)</th>
<th>Exposure Time (sec)</th>
<th>Average SNR</th>
<th>Fitted Diffusion Coefficient D (μm²/s)</th>
<th>Fitted PoL σ (μm)</th>
<th>Average # of Frames per Trajectory</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6</td>
<td>0.4</td>
<td>6.26</td>
<td>3.41×10⁻⁶</td>
<td>0.089</td>
<td>340</td>
</tr>
<tr>
<td>5.2</td>
<td>0.2</td>
<td>7.87</td>
<td>1.71×10⁻⁶</td>
<td>0.07</td>
<td>444</td>
</tr>
<tr>
<td>10.4</td>
<td>0.1</td>
<td>8.48</td>
<td>4.47×10⁻⁶</td>
<td>0.064</td>
<td>443</td>
</tr>
<tr>
<td>20.8</td>
<td>0.05</td>
<td>8.98</td>
<td>1.38×10⁻⁵</td>
<td>0.066</td>
<td>464</td>
</tr>
<tr>
<td>41.6</td>
<td>0.025</td>
<td>8.65</td>
<td>7.5×10⁻⁷</td>
<td>0.071</td>
<td>415</td>
</tr>
</tbody>
</table>

5.3.3 The distribution of receptors and G proteins in the cell

GFP-tagged M₂ receptors and mCh-tagged G₈ proteins were expressed in CHO cells and imaged with the TIRF microscope in Gradinaru Lab. Fluorescent particles of various sizes were observed in more than 30% of the cells imaged. Our previous estimation of the optical resolution for the TIRF setup was ~440 nm (as the 1/e radius of the 2D Gaussian image profile of a 20nm fluorescent bead), which was approximately 2.6 pixels under the current magnification at the camera. Therefore, any particles with diameter larger than 3 pixels were likely large complexes consisting of more than one molecule. Moreover, since the optical resolution was ~100 times larger than the size of the subject molecules, it is possible that the smaller fluorescent particles were also oligomeric complexes instead of single molecules. Therefore, the subsequent tracking analysis in this chapter was based on the motion of the complexes formed by one or more receptor and/or G protein molecules.
Figure 5-4. TIRF images of a CHO cell co-expressing GFP-M$_2$ and mCh-G$_i$. (A) The spatial distribution of the M$_2$ receptors was captured by illumination at 473 nm and detection of the GFP fluorescence. (B) The distribution of the G$_i$ protein was captured by illumination at 532 nm and detection of the mCherry fluorescence. (C) The distribution of mCh-G$_i$ protein was re-captured after treating the cell culture with an agonist ligand (10mM carbachol) that activated the M$_2$ receptor. The images have the same distance scaling, but different brightness scaling, which is determined by the brightest pixel in each image.

In Figure 5-4, it can be seen that the M$_2$ receptors and G$_i$ proteins showed different patterns of distribution in their inactive states. Once the M$_2$ receptors were activated by the binding of agonist, the spatial distribution of the G$_i$ protein was altered into a pattern that visually resembled the distribution of M$_2$ receptor (see panels A and C of Figure 5-4). Details of this co-localization were studied and presented in the following sections by analyzing different modes of lateral diffusion of the particles at the cell membrane.

5.3.4 Diffusion of GFP-M$_2$ meceptor

GFP-M$_2$ receptors were co-expressed with either mCh-tagged or wild-type G$_i$ proteins in CHO cells. Based on our previous findings with the dcFCS technique (Chapter 3.3), the receptors were expected to be mostly uncoupled from the G$_i$ proteins in the absence of the agonist ligand. TIRF movies of the motion of the basal-state (i.e. inactive) GFP-M$_2$ were recorded using blue (473nm) laser excitation. Fluorescent particles were identified and tracked using the TrackMate program. MSD functions were constructed for each individual trajectory, and fitted to the general anomalous diffusion model (Equation 5.5). The diffusion analysis results are shown in Figure 5-5.
Figure 5-5. Diffusion analysis of basal state (i.e. inactive) GFP-M₂ receptors tracked in live cells, in the absence of receptor ligands but in the presence of exogenous Gi proteins. A total of 503 trajectories were retrieved from approximately 150 particles in 3 cells. (A) The Time-Averaged MSD functions for all trajectories were computed (Equation 5.2) and fitted to the anomalous diffusion MSD Model (Equation 5.5). (B) All single-particle trajectories from a single cell were overlaid to a common starting point. The fitted diffusion coefficient for each trajectory was used as the color scale for Panels A and B. (C) The fitted anomalous factors were grouped in a histogram, with the vertical dashed lines indicating the boundary values assigned for free diffusion, α = 0.8 and α = 1.2. (D) The fitted diffusion coefficients were grouped in a histogram, with logarithmically scaled x-axis.

No sign of global directional bias in the motion of the tracked particles was observed in the trajectory overlay (Figure 5-5, Panel B), indicating that the tracking analysis of the M₂ receptors was not affected by the global cellular motions. In addition, rare but prominent directed receptor transport was observed among the majority of the slow or restricted diffusion trajectories.
Using the boundary values $\alpha=0.8$ and $\alpha=1.2$, the trajectories were categorized into three different types of behaviors: sub-diffusion ($\alpha < 0.8$), normal diffusion ($0.8 < \alpha <1.2$), and super-diffusion ($\alpha > 1.2$). A majority of normal or sub-diffusion, and a minority of super-diffusion trajectories were observed for the inactive $M_2$ receptors (Figure 5-5, Panel C).

The ensemble-averaged diffusion coefficient considering all particles under different modes of diffusion was determined to be $0.011 \, \mu m^2/s$. (Figure 5-5, Panel D). This value was within the same order of magnitude with the reported diffusion coefficients of the membrane receptors studied using SPT methods. For instance, $0.001–0.1 \, \mu m^2/s$ was measured for $\text{GABA}_{\alpha}R$ by Alcor et.al.[2], $0.001–0.01 \, \mu m^2/s$ for $\mu$-OR by Daumas et.al.[1], $0.001–0.1 \, \mu m^2/s$ for EGFR by Boggara et.al.[14], and $0.1–1 \, \mu m^2/s$ for TfR by Fujiwara et.al.[18]. Further analysis separating different diffusion regimes were carried out as described below in Sections 5.3.7–5.3.9.

5.3.5 Diffusion of mCh-$G_i$ protein

mCh-tagged $G_i$ proteins were co-expressed with either GFP-tagged or wild-type $M_2$ receptors in live CHO cells. The $G_i$ proteins were mutated with the N270D substitution in the $G\alpha$ subunit to ensure the formation of stable receptor-$G$ protein complex [19, 20]. The motion of the $G_i$ proteins without the influence of the receptor was first studied, where the $G_i$ proteins were still expected to be mostly uncoupled from the $M_2$ receptors in the absence of the receptor agonist. TIRF movies of the mCh-$G_i$ proteins were recorded using the green 532 nm laser excitation and then tracked by the 

TrackMate program. MSD functions were constructed for each individual trajectory, and fitted to the anomalous diffusion model (Equation 5.5). The results of this diffusion analysis are shown in Figure 5-6.
Figure 5-6. Diffusion analysis of the mCh-Gi proteins tracked in live cells, in the presence of inactive M2 receptors. A total of 350 trajectories were retrieved from approximately 150 particles in 3 different cells. (A) The Time-Averaged MSD functions for all trajectories were computed (Equation 5.2) and fitted to the anomalous diffusion model (Equation 5.5). (B) The trajectories from a single cell were overlaid to a common starting point. The fitted diffusion coefficient for each trajectory was used as the color scale for Panels A and B. (C) The fitted anomalous factors were grouped in a histogram, with the vertical dashed lines indicating boundary values assigned for free diffusion, $\alpha = 0.8$ and $\alpha = 1.2$. (D) The fitted diffusion coefficients were grouped in a histogram, with logarithmically scaled x-axis.

As for the receptors, no sign of directional bias was observed in the trajectory overlay (Figure 5-6, Panel B), indicating that the tracking analysis of the mCh-Gi proteins was not affected by the global motion caused by cellular activities. However, in contrast to the receptors, the directed transportation trajectories were displaying similar travel ranges as the trajectories which showed random diffusion.
Using boundary values $\alpha = 0.8$ and $\alpha = 1.2$ to separate different diffusion regimes, the mCh-G$_i$ proteins tracked in the presence of inactive receptors were found to exhibit a majority of normal or super-diffusion SPTs, and a minority of sub-diffusion SPTs (Figure 5-6, Panel C). Note that the receptors showed a predominance of normal and sub-diffusion behavior (Figure 5-5, Panel C).

The ensemble-averaged diffusion coefficient of the mCh-G$_i$ proteins in the presence of inactive M$_2$ receptors was determined to be 0.017 $\mu$m$^2$/s (Figure 5-6, Panel D), ~50% faster than the value obtained for the inactive receptors. Despite the lack of published SPT studies on the G proteins, the diffusion coefficient was comparable to those measured from the myristoylated/palmitoylated lipid anchored proteins (0.01~1 $\mu$m$^2$/s [21, 22]) or glycosylphosphatidylinositol-anchored (GPI-anchored) proteins (0.001~0.02 $\mu$m$^2$/s [23, 24]). A more in-depth analysis separating the G protein SPT data into different diffusion regimes is described in Sections 5.3.7–5.3.9.

5.3.6 Diffusion of the activated R-G complex, tracking G protein

In the CHO cells where mCh-tagged G$_i$ proteins were co-expressed with GFP-tagged or wild-type M$_2$ receptors, 10mM carbachol was introduced to the cell culture to activate M$_2$ receptors. The activated M$_2$ receptors were then recruited to and coupled with the G$_i$ proteins, forming activated receptor-G protein (R-G) complexes. TIRF movies of the activated R-G complex were recorded with the green (532-nm) laser excitation (observing the motion of the G$_i$ protein only) and then analyzed by the TrackMate program for SPT trajectories. MSD functions were constructed for each individual trajectory and fitted to the anomalous diffusion model (Equation 5.5). The diffusion analysis results are shown in Figure 5-7 below.
Figure 5-7. Diffusion analysis of the mCh-G_i proteins in the presence of active M_2 receptors, tracked in live cells after treatment with M_2 receptor agonist (10mM carbachol). A total of 407 trajectories were retrieved from approximately 100 particles in 2 cells. (A) The Time-Averaged MSD functions for all trajectories were computed (Equation 5.2) and fitted to the anomalous diffusion model (Equation 5.5). (B) The trajectories from a single cell were overlaid to a common starting point as an overview of behaviors. The fitted diffusion coefficient for each trajectory was used as the color scale for Panels A and B. (C) The fitted anomalous factors were grouped in a histogram, with the vertical dashed lines indicating boundary values assigned for free diffusion, \( \alpha = 0.8 \) and \( \alpha = 1.2 \). (D) The fitted diffusion coefficients were grouped in a histogram, with logarithmically scaled x-axis.

Compared to the diffusion of inactive G_i proteins (Figure 5-6), the diffusion of G_i proteins in the presence of activated M_2 receptors showed overall trajectories resembling those found on the M_2 receptors (Figure 5-7, Panel B). Using the values \( \alpha = 0.8 \) and \( \alpha = 1.2 \) to delineate the diffusion regimes, the mCh-G_i proteins tracked in the presence of activated receptors were found to exhibit an increased fraction of sub-diffusion, and
decreased contributions from both normal and super-diffusion (Figure 5-7, Panel C), and a smaller diffusion coefficient (Figure 5-7, Panel D) than the inactive/uncoupled G_{i}’s (Figure 5-6, Panel D). The ensemble-averaged diffusion coefficient was 0.010 µm^{2}/s, nearly identical to that found for the M_{2} receptors. These results provided insights into the mechanism of receptor-G protein interaction. In the absence of stimuli/ligands that could activate the receptor, G_{i} proteins appeared to be separated from the inactive M_{2} receptors, displaying distinctive diffusion behaviors. However, the recruitment of G_{i} proteins to the activated receptors altered the diffusion properties of the G_{i} proteins to resemble those of receptors, implying formation of co-diffusing (coupled) complexes.

It is notable that the diffusion coefficients of the membrane proteins measured by the SPT method were significantly slower than those measured by FCS (in Chapter 3). The diffusion coefficients of membrane receptors in live cells measured with FCS were usually in the range of 0.5~10 µm^{2}/s according to the literature and measurements within the lab [25-28]. This discrepancy could be understood by considering the different spatial and temporal scales of the two techniques. The SPT technique observes the motion of the particles across the area of a whole cell (10–50 µm), for a typical duration of ~10 sec per particle. In contrast, the FCS technique observes the motion of the particles inside a diffraction-limited detection volume (~0.5 µm) for a typical duration of 0.1–10 ms per particle (i.e. the diffusion correlation time). Consequently, the diffusion coefficients measured by FCS are mostly related to the motion in the local environment of the molecules, whereas the diffusion coefficients measured by SPT method are more sensitive to the cellular-scale factors such as crowding, confinement, or intracellular transportation.

In order to acquire physiologically relevant properties of the motion of the M_{2} receptors and G_{i} proteins, we separately analyzed each population of proteins undergoing sub-diffusion, super-diffusion, and normal diffusion. The relative proportions of the three types of diffusion found in the receptor and/or G protein samples that were estimated based on the data shown in Figs. 5-5, 5-6 and 5-7 (Panels C) are listed in Table 5-4.
Table 5-4. Relative proportions of the three diffusion regimes as inferred from the SPT analysis of M₂ receptors or Gᵢ proteins.

<table>
<thead>
<tr>
<th></th>
<th>Sub-diffusion (%)</th>
<th>Normal diffusion (%)</th>
<th>Super-diffusion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₂ (inactive)</td>
<td>30.9</td>
<td>43.5</td>
<td>25.6</td>
</tr>
<tr>
<td>Gᵢ (with inactive M₂)</td>
<td>26.9</td>
<td>38.0</td>
<td>35.1</td>
</tr>
<tr>
<td>Gᵢ coupled (with active M₂)</td>
<td>54.1</td>
<td>31.2</td>
<td>14.7</td>
</tr>
</tbody>
</table>

5.3.7 Confined diffusion regime: $\alpha < 0.8$

The particles that yielded anomalous factors $\alpha < 0.8$ on the MSD functions were further analyzed with a confined diffusion model (Equation 5.6) to explore the possible cause of sub-diffusion.

![Image of Figure 5-8](image)

**Figure 5-8.** Fitting of the MSD functions with anomalous diffusion model vs. confined diffusion model. Sixteen MSD functions were randomly picked from the sub-diffusing population of the GFP-M₂ samples. These MSD functions were fitted first with the anomalous diffusion model (Panel A, Equation 5.5), then with the confined diffusion model (Panel B, Equation 5.6).

Judging by a random selection of MSD functions (Figure 5-8), the confined diffusion model noticeably improved the quality of fitting. To quantitatively evaluate the goodness
of fit, Akaike Information Criterion (AIC) was computed after fitting the MSD functions to the anomalous or the confined diffusion model [29, 30]:

$$AIC = n \cdot \ln \left( \frac{wRSS}{n} \right) + 2k + \frac{2k(k + 1)}{n - k - 1}$$  \hspace{1cm} \text{Equation 5.8}

where \( n \) is the number of data points in each MSD curve, \( k \) is the number of parameters in the fitting model, and \( wRSS \) is the weighted sum of squares obtained from the least squares curve fitting process (MATLAB built-in function “lsqnonlin”). The fitting model that yields the lowest AIC value is statistically preferred over the other models under comparison. Each individually time-averaged MSD function was fitted first to the anomalous model (Equation 5.5), and then to the confined model (Equation 5.6), generating a pair of AIC values. All AIC value pairs from the sub-diffusing populations of the receptor and/or G protein samples are plotted in Figure 5-9.

**Figure 5-9.** Pair-wise comparison of the AIC values from the confined diffusion model (y-axis) versus those from the anomalous diffusion model (x-axis). Each data point is from a pair of AIC values obtained from fitting one individual time-averaged MSD function, among the sub-diffusing populations of (A) the inactive GFP-M2 receptor, (B) the mCh-Gi protein with inactive receptors, and (C) the mCh-Gi protein with activated receptors. The red lines (\( y = x \)) indicate the borderline for equal AIC values from both confined and anomalous models.

In Figure 5-9, most AIC value pairs are located below the \( y = x \) borderline. In other words, the confined diffusion model was the statistically preferred model over the anomalous model for fitting most of the MSD functions of the sub-diffusing particles. The confined diffusion model was therefore employed for interpreting the sub-diffusion observed among the M2 receptors and the Gi proteins.
The characteristic radius $r_c$ and the confined diffusion coefficient $D_c$ are the key parameters of the confined diffusion fitting model. A comparison of the $r_c$ and $D_c$ values retrieved from the sub-diffusing particles is provided in Figure 5-10.

![Figure 5-10](image)

**Figure 5-10. Spread histograms of the radius of confinement ($r_c$) and the diffusion coefficient ($D_c$).** The $r_c$ and $D_c$ values were retrieved by fitting the MSD functions of the trajectories showing sub-diffusion ($\alpha < 0.8$) to the confined diffusion MSD model (Equation 5.6). A comparison is made between the sub-diffusing populations of the inactive GFP-M2 receptor (“M2”), of the mCh-Gi protein with inactive receptors (“Gi”), and of the mCh-Gi protein with activated receptors (“Gi coupled”). The mean value and the uncertainty of the mean for each distribution are shown in red cross and red error bar, respectively.

The average radius of confinement $r_c$ and average confined diffusion coefficient $D_c$ shown in Figure 5-10 are summarized in Table 5-5. Although the precise nature of the confinement found in these experiments remains elusive, the characteristic radii of confinement found here (0.1–0.2 μm) are in agreement with the reported membrane compartment sizes [31, 32]. Two types of confinement can be found in the cell membrane: permeable confinement formed by lipid rafts (with sizes of 0.01–0.2 μm) [33, 34], and impermeable confinement defined by membrane skeleton domains bounded by the actin filament meshwork (with sizes larger than 0.1 μm)[35, 36]. Judging by the sizes derived by fitting, both confinement scenarios are compatible with the observed confined diffusion in our experiments.
Table 5-5. Summary of the confined model and anomalous model fitting results of the MSD functions of the particles displaying sub-diffusion.

<table>
<thead>
<tr>
<th>Sub-diffusion</th>
<th>M₂</th>
<th>Gᵢ</th>
<th>Gᵢ coupled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Radius of Confinement ( r_c ) (μm)</td>
<td>0.110 ± 0.004</td>
<td>0.23 ± 0.01</td>
<td>0.160 ± 0.006</td>
</tr>
<tr>
<td>(Confined Model)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Diffusion Coefficient ( D_c ) (μm²/s)</td>
<td>0.074 ± 0.003</td>
<td>0.076 ± 0.006</td>
<td>0.082 ± 0.007</td>
</tr>
<tr>
<td>(Confined Model)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Anomalous Factor ( \alpha )</td>
<td>0.622 ± 0.008</td>
<td>0.63 ± 0.01</td>
<td>0.590 ± 0.007</td>
</tr>
<tr>
<td>(Anomalous Model)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Diffusion Coefficient ( D_a ) (μm²/s)</td>
<td>0.0026 ± 0.0003</td>
<td>0.0059 ± 0.0006</td>
<td>0.0029 ± 0.0002</td>
</tr>
<tr>
<td>(Anomalous Model)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The difference in the mobility of the Gᵢ proteins induced by the receptor activation appears to be mostly an effect of reduced confinement area, rather than decreased diffusion coefficient (Table 5-5). The impermeable actin meshwork confinement scenario could explain this effect. From a microscopic point of view, this would mean that the coupling of G protein to the activated receptors increased the chance for the signaling complex to collide with the actin meshwork, resulting in a smaller effective confinement area [23, 37]. On the other hand, the diffusion coefficient of the receptor within the confinement region was largely unaffected by the G protein coupling, because the diffusion of the receptors in the unobstructed membrane would be mostly determined by their transmembrane domains, which would remain mostly unchanged upon the agonist activation and the G protein coupling [38, 39].

5.3.8 Directed transportation: \( \alpha > 1.2 \)

The most likely cause of the super-diffusion in live cells is the active transport. In order to study the average speed of the transportation, speed analysis was performed on the trajectories that showed MSD functions with anomalous factor \( \alpha \) larger than 1.2. For each...
tracked particle, the root-mean-square displacement (RMSD) function was calculated as the square root of the MSD function. The RMSD as a function of the lag time \( \tau \) was then fitted to a linear model:

\[
RMSD(\tau) \equiv \sqrt{MSD(\tau)} = v_{RMS} \tau
\]

Equation 5.9

where \( v_{RMS} \) was interpreted as the root-mean-square speed (RMS speed) of the trajectory. A comparison of the RMS speed distributions retrieved from super-diffusing particles of inactive M\(_2\) receptors, uncoupled G\(_i\) proteins, and receptor-coupled G\(_i\) proteins is shown in Figure 5-11.

![Spread histograms of the root-mean-square (RMS) speed.](image)

**Figure 5-11. Spread histograms of the root-mean-square (RMS) speed.** The RMS speed was computed by fitting the MSD functions of the trajectories showing super-diffusion (\( \alpha > 1.2 \)) to Equation 5.9. Comparison is made between the super-diffusing populations of the inactive GFP-M\(_2\) receptor ("M\(_2\)"), of the mCh-G\(_i\) protein with inactive receptors ("G\(_i\)"), and of the mCh-G\(_i\) protein with activated receptors ("G\(_i\) coupled"). The mean value and the uncertainty of the mean for each distribution were shown in red cross and red error bar, respectively.
Table 5-6. Summary of the RMS speed analysis performed on the particles displaying super-diffusion and of the anomalous MSD fitting parameters for the same particles.

<table>
<thead>
<tr>
<th>Super-diffusion</th>
<th>$M_2$</th>
<th>$G_i$</th>
<th>$G_i$ coupled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average RMS speed $v_{RMS}$ (μm/s)</td>
<td>0.108 ± 0.004</td>
<td>0.169 ± 0.005</td>
<td>0.137 ± 0.006</td>
</tr>
<tr>
<td>Average Anomalous Factor $\alpha$ (Anomalous Model)</td>
<td>1.41 ± 0.02</td>
<td>1.49 ± 0.02</td>
<td>1.52 ± 0.04</td>
</tr>
<tr>
<td>Average Diffusion Coefficient $D_a$ (μm$^2$/s) (Anomalous Model)</td>
<td>0.020 ± 0.001</td>
<td>0.0111 ± 0.007</td>
<td>0.0062 ± 0.0005</td>
</tr>
</tbody>
</table>

The super-diffusion behavior of membrane receptors has been attributed to the transportation of the nano-complexes along the actin meshwork [40]. The class V and VI myosins have been reported to be responsible for the transportation of cargo at the actin-rich periphery of cells [41, 42]. The maximum transportation speeds of class V and class VI myosin were both estimated to be ~0.2 μm/s [43]. The speed of active transportation observed in our experiments was therefore consistent with the active transport speed by molecular motors along the actin cytoskeleton in the proximity of the cell membrane.

5.3.9 Free diffusion: 0.8 $< \alpha <$1.2

For the trajectories that did not significantly show either sub-diffusion or super-diffusion, the MSD functions were fitted to the normal, Brownian diffusion model (Equation 5.4). The fraction of free normal diffusion (30-45%, Table 5-4) was not expected to be so high for a membrane protein. Several factors could cause this effect. First, the receptors and the G proteins could be diffusing in domains on the membrane that were not restricted by the actin barriers. Second, it was also possible that a fraction of the transfected proteins did not complete the expression process or did not reach the cytoplasmic membrane, thus appearing to be diffusing in the cytoplasm, near the membrane. Last but not the least, particles switching between confined diffusion and directed transportation may result in MSD functions that could be misinterpreted by the anomalous model (Equation 5.5), resulting in an “effective” anomalous factor close to 1.
Figure 5-12. Spread histograms of the diffusion coefficient $D_n$. The $D_n$ values were retrieved by fitting the MSD functions of the trajectories with $0.8 < \alpha < 1.2$ to the normal diffusion MSD model (Equation 5.4). A comparison is made between the normal diffusion populations of the inactive GFP-M$_2$ receptors (“M$_2$”), of the mCh-G$_i$ proteins with the inactive receptors (“G$_i$”), and of the mCh-G$_i$ proteins with activated receptors (“G$_i$ coupled”). The mean value and the uncertainty of the mean for each distribution was shown in red cross and red error bar, respectively.

Table 5-7. Summary of fitting parameters of the normal diffusion model and the anomalous model for particles displaying normal diffusion behavior.

<table>
<thead>
<tr>
<th>Normal Diffusion</th>
<th>M$_2$</th>
<th>G$_i$</th>
<th>G$_i$ coupled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Diffusion Coefficient $D_n$ (μm$^2$/s)</td>
<td>$0.0103 \pm 0.0007$</td>
<td>$0.028 \pm 0.002$</td>
<td>$0.0238 \pm 0.002$</td>
</tr>
<tr>
<td>(Normal Model)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Anomalous Factor $\alpha$ (Anomalous Model)</td>
<td>$0.991 \pm 0.007$</td>
<td>$0.99 \pm 0.01$</td>
<td>$0.977 \pm 0.009$</td>
</tr>
<tr>
<td>Average Diffusion Coefficient $D_a$ (μm$^2$/s)</td>
<td>$0.0063 \pm 0.0003$</td>
<td>$0.0079 \pm 0.0006$</td>
<td>$0.0063 \pm 0.0004$</td>
</tr>
<tr>
<td>(Anomalous Model)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.10 Estimation of particle sizes via initial fluorescence intensity

In addition to the diffusion analysis, we performed fluorescence intensity analysis on the SPT trajectories of M₂ and G₁ particles to infer the number of monomers inside a tracked particle. The change-point analysis developed for the studies in Chapter 4 was not suitable for this purpose, because the majority of intensity traces of the tracked molecules did not fully photobleach and lacked distinctive step-like intensity drops (Figure 5-13).

![Figure 5-13](image)

**Figure 5-13.** Four representative intensity-time traces selected from tracking the GFP-M₂ in a live CHO cell.

A simple, initial brightness analysis was performed instead, in which the initial fluorescence intensities of the tracked particles were compared to that of the single fluorophore controls. In practice, the initial intensity of GFP-M₂ was compared with that of the membrane anchored monomeric MP-GFP’s; and the initial intensity of mCh-G₁ protein was compared with that of the membrane anchored monomeric MP-mCh’s. When
scaled to the same excitation laser power, the ratio of intensities provided an estimation of the number of fluorophores, *i.e.*, the oligomeric size of the particles.

### Table 5-8. Initial fluorescence intensities of M₂ receptor and Gᵢ protein samples, in comparison with the monomeric control samples.

<table>
<thead>
<tr>
<th></th>
<th>Average Initial Fluorescence Intensity per Particle (kcps/mW *)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-GFP</td>
<td>65 ± 2</td>
</tr>
<tr>
<td>GFP-M₂</td>
<td>176 ± 60</td>
</tr>
<tr>
<td>MP-mCh</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>mCh-Gᵢ</td>
<td>257 ± 70</td>
</tr>
</tbody>
</table>

*: “kcps” indicates kilo-counts per second. The value is in electron counts as directly reported by the EMCCD camera. The acquisition settings of the camera (namely the pre-amplification gain, the EM Gain, and the exposure time) for all four samples were kept the same. The power of the laser was measured before the microscope objective.

With the assumptions that the distribution of MP-GFP and MP-mCh on the surface of the CHO cell was not affected by the membrane confinement and was thus mostly monomeric, the ratio of the initial brightness of the tracked particles could then be used to estimate the number of fluorophores inside one detected particle. The M₂ receptor particles were thus estimated to be composed of two to four monomeric units, whereas the Gᵢ protein particles were estimated to contain four to eight heterotrimeric G proteins. These estimates were in agreement with the outcome of smPB measurements performed on purified and immobilized samples solubilized in detergent micelles, which were described in Chapter 4 [15].

### 5.3.11 SmPB measurements of M₂ oligomeric size in fixed cells

The difficulty in reliably tracking the moving particles is one of the major sources of uncertainty for the intensity-based estimation of the oligomeric sizes. The motion of the particles could be halted by fixing the cells, facilitating stable photobleaching
measurement of the immobilized particles in cell. The CHO cells expressing GFP-M$_2$ were fixed by incubating with 4% paraformaldehyde for 10 minutes at 4 °C. Among the cells studied, two cells were found with suitable levels of expression for the smPB method. The intensity change-point analysis program developed for immobilized molecules (Chapter 4.2.8) was employed to retrieve the average number of fluorophores per particle.

Figure 5-14. (A) TIRF image of a fixed cell showing immobile GFP-M$_2$ particles, averaged over a 100 sec measurement. (B) smPB histogram of the number of photobleaching steps (i.e., M$_2$ monomers) per particle.

The distribution of the number of photobleaching steps per particle (Figure 5-14 B) indicated the presence of 4–8 fluorophores per particle, in agreement with the estimations based on the initial fluorescence intensity in live cells (Section 5.3.10). Several issues remain to be resolved: the tight requirements for the expression level in order to perform immobilized smPB analysis might create a bias towards a certain particle size; the noise in the photobleaching intensity trace was still significant (Figure 5-15), preventing consistent and reliable change-point identification. An ongoing collaboration project with Dr. Andrew Rutenberg’s computational biophysics group (Dalhousie University) will address these issues based on the Bayesian analysis of the stochastic noise properties of the intensity trace [44].
Figure 5-15. A representative photobleaching intensity trace from a particle in a fixed cell expressing GFP-M2. The change-point analysis identified five PB steps despite only one distinct step-like intensity drop.

5.4 Conclusions

Repeated on no more than five different cells across three preparations, the conclusions of this study could be limited by the cell-to-cell variations and batch-to-batch variations. Nonetheless, the results were leading to some interesting aspects related to the distribution, the mobility, and the interaction of the M₂ receptors and the Gᵢ proteins in the membrane of live cells.

**Significant presence of the sub-diffusion and the super-diffusion regimes.** Both of the M₂ receptors and the Gᵢ proteins exhibited a highly heterogeneous diffusion behavior, with fractions of the total population undergoing sub-diffusion (anomalous factor smaller than 1), super-diffusion (anomalous factor greater than 1), or normal diffusion (anomalous factor close to 1).

**Sub-diffusion: confinement due to the actin meshwork.** The M₂ receptor and Gᵢ protein particles that displayed sub-diffusion trajectories could be explained with the
confined diffusion model. The confinement radius was within the range of 100-200 nm and it was likely imposed by the actin meshwork on the membrane skeleton.

**Super-diffusion: active transportation.** The M\(_2\) receptor and G\(_i\) protein particles that displayed super-diffusion trajectories could be explained with the active transportation model. The root-mean-square speeds of the super-diffusing particles were compatible with the transportation speed of intracellular cargo by class V and VI myosin on actin filaments of the cytoskeleton.

**Inactive receptors diffuse slower than uncoupled G proteins.** In the absence of the agonist ligand, the diffusion of the inactive M\(_2\) receptor was consistently slower than that of the (uncoupled) G\(_i\) proteins. For the sub-diffusion population, the slower diffusion was due to a smaller confinement area, rather than a slower diffusion coefficient. The smaller confinement area for the receptor was possibly due to the more frequent collisions with the confinement barrier formed by the actin cytoskeleton.

**G proteins are recruited to the receptors after receptor activation.** Upon receptor activation by the agonist ligand, the diffusion of the G\(_i\) proteins became significantly slower. The coupling between M\(_2\) receptors and G\(_i\) proteins was therefore not spontaneous but induced by the receptor agonist.

### 5.5 Future Directions

Cholesterol Oxidase (COase) and sphingomyelinase (SMase) will be introduced to the cell culture to break down the formation of lipid rafts [32]. Then the spatial distribution and the diffusion behaviors of the receptors and the G proteins will be examined to clarify the effect of lipid rafts on the mobility and the activation of the M\(_2\) receptors and the G\(_i\) proteins.

An actin staining reagent [36, 45] will be introduced to the cell culture to reveal the membrane skeleton-actin meshwork confinement and elucidate its role on the confined diffusion and the active transport of GPCRs.
Two-color simultaneous tracking will be performed on the GFP-M$_2$ receptor and mCh-Gi proteins to directly estimate the fraction of G protein that are bound to receptors under different ligand conditions.

A new intensity trace analysis algorithm based on Bayesian information theory [44] will be applied to the fluorescence intensity traces of the tracked particles, providing more robust estimations of the oligomeric sizes of proteins in live cells.

Camera-based Total Internal Reflection Fluorescence Correlation Spectroscopy (TIR-FCS) [46, 47] will be developed as a supplementary routine to analyze the diffusion properties of the fluorescently labeled molecules in live cells. As a fluctuation based analysis method, the TIR-FCS does not require high signal-noise-ratio. For the cells that are not suitable for SPT, TIR-FCS can serve as an alternative method to extract locally averaged diffusion information.

5.6 References


Statement of Contributions

Chapter 2 (fluorescence techniques):

I was in charge of the design, construction, upgrade, and daily maintenance of the dcFCS microscope in the Gradinaru lab. I developed the data acquisition program in LabVIEW. Gregory Gomes developed the single-color FCS fitting program in MATLAB. I developed the dcFCS global fitting program based on the single-color FCS fitting program. I designed a standard dual-color DNA sample for calculating the overlap volume correction factor. I implemented the correction procedures for spectral bleed-through and detection volume overlap. I supervised an undergraduate student, Charles Huston, to design, build, and test the two-photon excitation (TPE) mode for the dcFCS microscope.

Chapter 3 (correlation studies):

The in vitro sample preparation and quality control tests were completed by Rabindra Shivnaraine, Tina Ji, and Fei Huang in the Wells lab. I performed the dcFCS experiments in detergent solution in the Gradinaru lab. For handling the live-cell samples, I was first trained by Rabindra Shivnaraine, and then I was responsible for maintaining the CHO cell culture, transfecting the cells with DNA plasmids, and performing the dcFCS experiments in live cells either in the Gradinaru lab or in the Imaging Facility at Hospital for Sick Children, Toronto. I performed analysis on all the data collected from the samples in solution and the samples in live cells. This work is currently being prepared for publication, for which I will be the first author.

Chapter 4 (photobleaching studies):

I implemented the surface passivation and immobilization protocol based on published literature. I adopted and improved the assembly protocol for the coverslide-based flow-chamber. The sample preparation and quality control tests were completed by Rabindra Shivnaraine, Tina Ji, and Fei Huang in the Wells lab. I performed the immobilization and observed the photobleaching of the monomeric GFP control sample on the TIRF microscope in the Gradinaru lab. Subsequent smPB measurements of GFP multiplexes,
M₂ receptors, and Gᵢ proteins were performed by Dennis Fernandes, who was at the time an undergraduate student under my supervision. The MATLAB image analysis programs for identifying single particles and extracting intensity traces were developed by Rabindra Shivnaraine. The MATLAB change-point analysis algorithm for identifying photobleaching steps was developed by Zhenfu Zhang. I merged and fine-tuned the two programs for specifically analyzing the smPB traces from GFP-tagged receptor and G protein samples. The processing of the smPB data through the analysis programs was completed by Dennis Fernandes. I am a co-author on the JACS paper published based on this work in 2016.

**Chapter 5 (tracking studies):**

I maintained the CHO cell line, transfected and expressed the fluorescent labeled M₂ receptors and Gᵢ proteins in CHO cells, and collected image sequence (movies) of mobile fluorescent particles in live cells on the TIRF microscope in the Gradinaru lab. Rabindra Shivnaraine initiated the collaboration with Kevin Braeckmans Group in Ghent University and obtained the tracking program that was developed by the Braeckmans Group. I supervised an undergraduate student, Mohamed Salama, who compared the performance of several other open-source tracking programs with the tracking program from Breackmans Group. TrackMate was eventually employed as the program for tracking all particles in our TIRF image sequences. I developed MATLAB programs for the calculation of mean squared displacement (MSD), and for the fitting of the MSD curves to various diffusion models. I performed the analysis of all the image sequences. This work is currently being prepared for publication, for which I will be the first author.

**DNA ladder project:**

In addition to the GPCR projects mentioned above, I have been in collaboration with ACGT corp. Toronto on a project to construct and characterize multi-step DNA ladder fluorescent probes. In this project, ACGT corp. synthesized fluorophore-tagged DNA oligonucleotide samples with various sequence design. I optimized the hybridization conditions and performed the construction of DNA ladders with the provided oligonucleotides. I performed smPB measurements and FCS measurements on the DNA
ladders. I optimized the measurement conditions to accurately and reliably quantify the number of steps per ladder molecule. The collaboration was funded by the OCE VIP1 NSERC Engage Grant.
List of Publications

   (In preparation)

   (In preparation)

   (To be submitted to *Biophysical Journal*, September 2017)


6. Fernandes D.D., Bamrah, J., Kailasam, S., Gomes, G.-N.W., **Li, Y.**, Wieden, H.-J., Gradinaru, C.C. To FLAsH or not to FLAsH? Characterization of Fluorescein Arsenical Hairpin (FLAsH) as a Probe for Single-Molecule Fluorescence Spectroscopy  
   (Accepted with minor revisions, *Nature Scientific Reports*, August 2017)