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Screening Next-generation Fluorine-19 Probe and Preparation of Yeast-derived G Proteins for GPCR Conformation and Dynamics Study

by

Wenjie Zhao

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Cell Biology, Microbiology and Molecular Biology
Colleague of Arts and Sciences
University of South Florida

Major Professor: Libin Ye, Ph.D.
Gary Daughdrill, Ph.D.
Jianfeng Cai, Ph.D.

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July 7th, 2021

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In addition, I would like to thank my Graduate Committee members: Profs. Gary Daughdrill, and Jianfeng Cai, who provided invaluable guides during my study. I would also express my great gratitude to my colleague Xudong Wang who worked with me together for parts of these projects, along with others, Aidan McFarland, Sriram Sundar Shankara Narayanan, Rana Hanna, Sheetal Vasoya, Kun Yan, and Daniel Chait. I was really enjoying the time that I spent with all of you in the past two years.

Dedication

This thesis work is dedicated to my roommate, Zhenhao Li, who has been a constant source of support and encouragement during the challenges of graduate school and life. I am truly thankful for having you in these two years. This work is also dedicated to my parents who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

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Abstract

GPCR regulates numerous diverse physiological processes relevant to diabetes, obesity, Alzheimer's diseases, and several central nervous system disorders and targets proteins in signaling pathways. It has created nearly 200 billion profits from its derivative drugs in 2018. There are near 400 structures of over 70 GPCRs have been resolved by X-ray crystallography, cryo-electron microscopy, and NMR spectroscopy. One of the current challenges that remain in the conformational transition and dynamics study using NMR spectroscopy is to obtain sufficient quantities of the G proteins and GPCRs. *Pichia pastoris has shown its tremendous promise in expressing the GPCRs in a high yield, along with isotopic labeling capacity for NMR studies. However***,** additional work is needed to reach the quantity requirement for most of the receptors. On the other hand, the mammalian system and insect cell expression systems require a higher cost of producing G proteins and GPCRs and it is still challenging for isotopic labeling. In this regard, this thesis is focused on developing and optimizing an applicable *P. pastoris* expression system for G proteins particularly to reach certain quantities required for NMR studies. In the meantime, a fluorine-19 screening was also performed to search for a next-generation fluorine-19 tag in probing GPCR conformational states at a higher resolution than the current state-of-the-art probe BTFMA. Through two-year work, we successfully established platforms for preparing yeast-derived G proteins, including Gα and Gβγ, which should be sufficient to conduct NMR studies. Though we are still exploring a next-generation ^{19}F probe, the current screening has given us several promising hits.

Chapter One: Next Generation ^{19}F probe Screening for GPCR Conformational Profiling

1.1 Introduction

GPCR regulates numerous diverse physiological processes relevant to diabetes, obesity, Alzheimer's diseases, and several central nervous system disorders and targets proteins in signaling pathways, already create nearly 200 billion profits from its derivative drugs in 2018. [1-3] GPCRs are a large family of cell surface receptors that play various roles in the signaling pathways. As their name indicates that GPCR can interact with G proteins triggered by a conformational change upon an extracellular molecule binding. The initial signal received by GPCR will pass to heterotrimeric G protein, which results in conformational changes of G proteins, allowing the nucleotide exchange with a consequence of dissociation between the G_{α} and the $G_{\beta\gamma}$ subunits. [4-6] There are two principal signal transduction pathways involving the G protein-coupled receptors: G protein and β-arrestin. [7, 8] GPCR can be characterized based on sequence and function: Class A – rhodopsin-like receptors, Class B-secretin family, Class C-metabotropic glutamate receptors, Class D-fungal mating pheromone receptors, Class E-cAMP receptors, and Class F-frizzled (FZD) and smoothened (SMO) receptors. [9] The adenosine A_{2A} receptor belongs to Class A and has been chosen since it's a prototypical class A G protein-coupled receptor. [10] The corresponding G protein subunit is $G\alpha_s$. [6, 11] Radioligand binding assay is a common method we use to determine the activity of receptors by measures the affinity of the isotopic labels ligand for a receptor and it has been used here to measure the activity of G protein base on the change of activity of the receptor. [12]

With the advancement of X-ray crystallography and cryo-electron microscopy (cryo-EM), so far, there are over 400 structures of 70 GPCRs have been resolved. [13, 14] . These resolved three-dimensional structures have dramatically promoted drug designing and discovery. [15] Recent publications revealing structures of GPCRs by X-ray as well established the foundation of the molecular mechanism of GPCRs' activation, facilitating the structure-based drug discovery and providing more reliable models for many other ligand-mediated GPCRs. [16] The structure of the adenosine A_{2A} receptor revealed by X-ray has successfully been elucidated with the extracellular loop (ECL2) included. [15] However, the difference while using X-ray or NMR spectrum in both binding mode and location of the adenosine A_{2A} receptor-ligand suggests that there may be still having limitations on the direct use of X-ray structures as templets for GPCRs. [15, 16] X-rays can determine the structures of GPCRs while the Cryo-EM can determine GPCRS coupled to either heterotrimeric G proteins ($G\alpha_i$, $G\alpha_s$ or $G\alpha_o$) or mini-G proteins (mini-$G\alpha_s$ or mini-$G\alpha_o$). [13, 17] However, both X-ray and cryo-EM were unable to elucidate the dynamics of protein-protein interactions or individual proteins. In this regard, Nuclear Magnetic Resonance (NMR) Spectroscopy is one of the most powerful biophysical tools. [18, 19] Base on this theory, one of our experiments takes advantage of the change of the resonance frequency which can give details of the electronic structure of sample molecules. It has been reported that the ^{19}F is the third most sensitive NMR nucleus to the microenvironmental changes, right after tritium and ^{1}H. [20] [21] To compare the chemical shift differences of different compounds, the NaF is used as a reference for free ^{19}F-tag screening. [13, 19] So far, BTFMA (2-Bromo-N-(4-(trifluoromethyl)phenyl) acetamide) is the most sensitive tri-fluorinated ^{19}F-tag used for profiling receptor confirmation with the state-of-the-art resolution, but the overlapping of different conformational states is still

an existing issue, which inspired us to initiate this project to **screen the next-generation ^{19}F-tag with a higher resolution**. [22, 23]

To study the conformation and dynamics of GPCRs, this study is to compare the chemical shift sensitivity of cysteine-specific trifluoromethyl probes in free tags states. The previous study indicated that it was able to use ^{19}F signal viability of a trifluoromethyl chemical equilibrium to probe the domain rotation in GPCR based on its sensitivity to microenvironmental changes, including the change due to receptor conformational change from ligand binding. [22-24] It also indicates the V229C on transmembrane domain VI (TM6) of adenosine A_{2a} receptor (as shown in Figure 1) is a desirable labeling site. [10, 25] Since the use of probes with relatively small chemical shift anisotropy (CSA) is important to the study of protein states in ^{19}F NMR studies. [22, 25] In this study, trifluoromethyl chemicals were predicted based on their molecular structures comparing to the BTFMA. [22, 25] We were anticipating obtaining a ^{19}F probe better than the current BTFMA tag in probing the GPCR conformational states with a higher resolution by exhibiting effective chemical shift dispersion. The selected ones in free tag screening will be verified via labeling adenosine A_{2A} receptors by conjugating to the site of V229C.

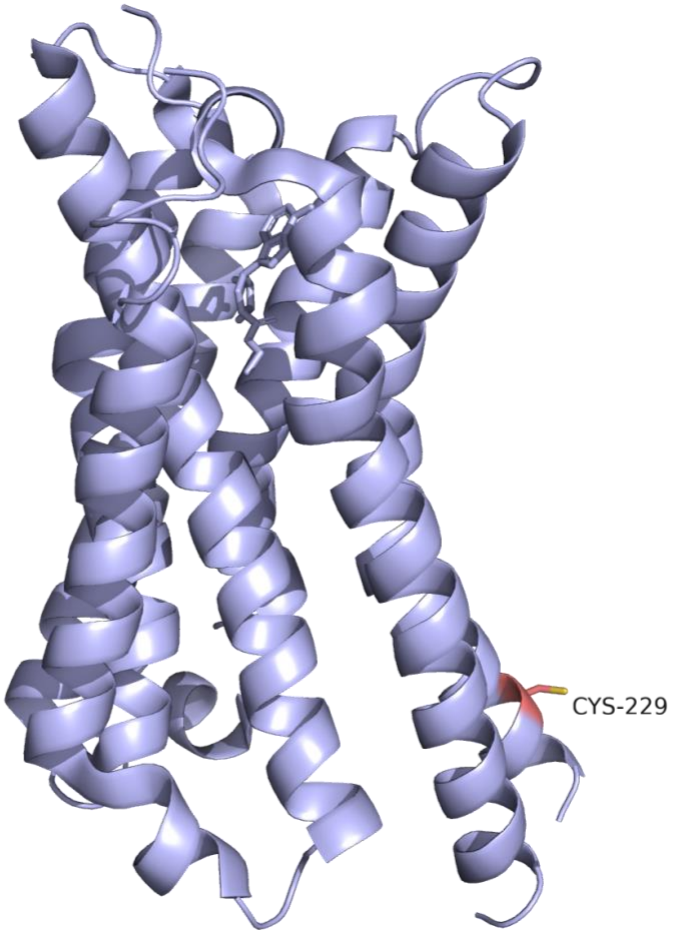


Figure 1. GPCR structure: A_{2A} adenosine receptor with V229C

1.2.1 $A_{2A}R$ expression and purification

The construct of pPIC9K_ADORA2A was kindly provided by T.Kobayashi (Japan), which was a recombinant vector containing the ADORA2A receptor sequence. [26] A pre-cultured single colony derived stock spread on YPD (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose) plates containing 1 mg/mL G418 was inoculate into 4 mL YPD medium and cultured at 30 °C overnight with shaking (275 rpm). Then, transferred into 200 mL BMGY (1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB (yeast nitrogen base) without amino acids, 0.00004% (w/v) glycerol and 0.1 M PB (phosphate buffer) pH 6.5) medium in 14 mL Falcon tubes at 30 °C

for at least 24 h with shaking (275 rpm) until the absorbance of A_{595nm} in the range of 2 to 6. The medium was then transferred into 1 L BMMY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB (yeast nitrogen base) without amino acids, 0.00004% (w/v) biotin, 0.1 M PB (phosphate buffer) pH 6.5, 0.04% (w/v) histidine, 3% (v/v) DMSO and 10 mM theophylline) at 20 °C for 60 h with shaking (275 rpm). Methanol (0.5% (v/v)) was added into the medium every 12 h to induce the adenosine A_{2A} receptor expression. [23, 27] 10x phosphate buffer (49.7 g Na_2HPO_4 and 98.0 g NaH_2PO_4 in 10000 mL D_2O, pH 6.5) was prepared in ahead.

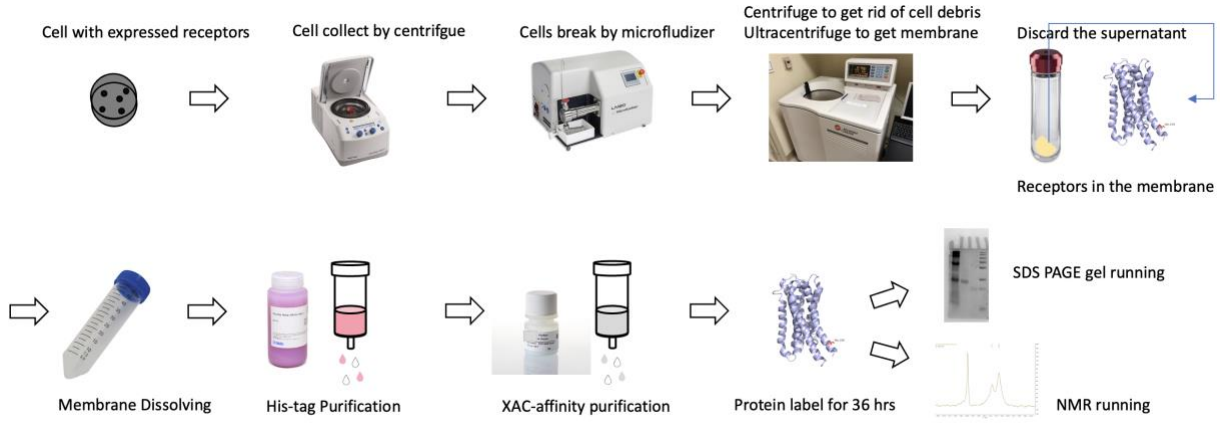


Figure 2. Purification and labeling flowchart for adenosine A_{2A} receptor

As shown in Figure 2, the cell pellets were collected by centrifuge at 3,800 × g for 10 min at 4 °C in 400 mL centrifuge bottles and then washed with Common washing buffer P1 (20 mM Bis-Tris, pH 6.5 or 50 mM HEPES, pH 7.4). Then the cell pellets were resuspended into Receptor Lysis Buffer P2 (20 mM Bis-Tris, pH 6.5 or 50 mM HEPES, pH 7.4, 2.5 mM EDTA, 10% Glycerol) at a ratio of four times the volume of cell pellets itself. The cells in P2 were broken via Microfluidizer for four cycles on the ice at 15,000 psi and centrifuge at 9,720 × g for 30 min at 4 °C in 250 mL centrifuge bottles to get rid of cell debris. The membrane supernatant was collected and centrifuged at 100,000 × g for 75 min using T45 rotor for the Beckman Ultracentrifuge, and the precipitated

cell membrane was then immediately dissolved in Receptor Preparation Buffer P3 (20 mM Bis-Tris, pH 6.5 or 50 mM HEPES, pH 7.4, 100 mM NaCl, 1% MNG-3 and 0.02% CHS (cholesterol hemisuccinate)) at 4 ℃ with shaking until dissolved sufficiently. [27, 28]

The dissolved membranes were centrifuged at 1,980 × g for 5 min at 4 ℃ to get rid of undissolved ones and then bind to Receptor preparation washing buffer P4 (20 mM Bis-Tris, pH 6.5 or 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% MNG-3, 0.002% CHS) pre-balanced Talon Resin for 2 h at 4 ℃ with shaking. The amount of resin usage for a 6 g membrane should be around 2 mL. Then, the Talon Resin can be packed onto a column and performed His-tag purification. First, using Receptor preparation washing buffer P4 wash on column 5 column volumes and then eluted the expressed protein, which supposed to be adenosine A_{2A} receptor (35.1 kDa), strategically recycling using 10 mL Column Elution Buffer P5 (20 mM Bis-Tris, pH 6.5 or 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% MNG-3, 0.002% CHS and 300 mM Imidazole) at a gravity rate. [29] Use Ultra-15 Centrifuge filters 3K to concentrate the collected eluted receptors to 1 mL and wash the sample once with 10 mL Receptor preparation washing buffer P4 at 4 ℃ at 3,846 × g. The concentrated sample goes through Receptor preparation washing buffer P4 pre-balanced XAC ligand column three times. The XAC column was washed with 2 column volume Receptor preparation washing buffer P4. Elute receptor with 8 mL Receptor preparation washing buffer P4 containing 25 mM theophylline, which is also called XAC Column Elution Buffer P6. The first 2 mL XAC Column Elution Buffer were eluted directly, while the rest of them needs incubation for 20 min before elution. Use Ultra-15 Centrifuge filters 3K to concentrate the collected eluted receptors to 1 mL and wash the sample once with 10 mL Receptor preparation washing buffer P4 at 4 ℃, 3,846 × g.

1.2.2 ^{19}F NMR experiments for free tri-fluorinated compounds

To evaluate the sensitivity of each potential tag to microenvironmental changes, a series of methanol/water mixtures were prepared with a range from (MeOH: H_2O = 4) to (MeOH: H_2O = 1/4) to create a polarity screening system, mimicking micro-electrostatic environment. [22, 23] NMR experiments were performed on a 600 MHz spectrometer with a ^{19}F spectroscopy probe. Chemical shift sensitivity was measured at 20 °C with reference NaF at (-119 ppm) and spectra were collected and processed by MestReNova. [25, 30]

1.2.3 ^{19}F NMR experiments on ^{19}F labeled $A_{2A}R$ receptor

The previous study revealed that the BTFMA tag exhibited a significantly greater range of chemical shifts as a function of solvent polarity. [31] BTFMA use as the sample of NMR labeling at a final concentration of 1 mM here. The mixture of Talon Resin containing expressed receptors was conjugated with the BTFMA label and incubated at 4 °C with gently shaking for 36 h via adding BTFMA every 12 h to ensure the labeling efficiency before performing standard His-tag purification as described in 1.2.1 [12, 23]. The ^{19}F NMR experiments were conducted at 20°C on the DD600 variant Spectrometer equipped with a ^{19}F probe.

1.2.4 SDS-PAGE

Preparing the SDS-PAGE gel base on the size of protein and our choice is 12% for separating and 6% for stacking gel. The purified protein mixture to the 2 × loading (50 mL receipt: 1 mg bromophenol blue, 12.5 mL 1.5 M Tris-HCl, pH 6.8, 2.5 mL10% SDS (sodium dodecyl sulfate) and 5 mL 100% glycerol) at 1:1 ratio. Load the sample onto the gel with PageRuler™ Prestained Protein Ladder and run at 120 V for 90 min. The SDS-Page running buffer was prepared as 25 mM Tris-base, 192 mM glycine, and 0.01 % SDS.

Fix the gel in 50% (v/v) methanol and 10% (v/v) glacial acetic acid for 5-10 min and then stain the gel in 50% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.1% (v/v) Coomassie brilliant blue R-250 for at least 30 mins. Destain the gel with 40% (v/v) methanol and 10% (v/v) glacial acetic acid and then visualize the gel using Bio-rad ChemiDoc system.

1.2.5 Radioligand binding assay for ^{19}F compound labeled $A_{2A}R$

During the preparation of the adenosine A_{2A} receptor described in 1.2.1, took some supernatant right before the ultracentrifuge step as the $A_{2A}R$ membrane sample and the purified protein as the $A_{2A}R$ protein sample. Incubate a certain number of labeled proteins based on its concentration measured by BCA assay with binding buffer (25mM pH 7.4 HEPES, 100mM NaCl) and a series of hot ligands (3H-CGS21680 for $A_{2A}R$) for the experimental group, additionally with cold ligand with a final concentration of 2 μM to ensure the hot ligand competitor been replaced out for the control group. The experimental group has the same setting except of replacement of the cold ligand in the control group with the same volume of DMSO. [32] The series hot ligands normally should be continuing from 0.5 nM, 1 nM, 2.5 nM, 5 nM, 7.5 nM, and 10 nM of CGS21680, but as well depends on the saturation curve of assessment. [10, 33] We usually use 50 μL as a total volume and six different concentrations of hot ligands as data points. Incubate the mixture at room temperature for 2 hours. Of note, the filter paper was pretreated with 0.2% polyethyleneimine PEI. Pre-wash the membranes with 2 mL binding buffer, vacuum dry and then, load the sample on and vacuum dry. Wash the sample-loaded membrane via vacuum with three times 4 mL binding buffer. Measure the radioactivity by soaking the membranes in scintillation liquid via a Beckman Coulter LS6500 liquid scintillation counter (LSC) immediately, repeat three times, and SD values were calculated from these measurements. [12]

1.3 Result and Discussion

Using BTFMA labeled $A_{2A}R$ as a model, the expression and purification of $A_{2A}R$ were confirmed by the SDS gel as shown in Figure 3. There were three lanes shown in the figure: $A_{2A}R$ control, protein after His-tag purification, protein after XAC affinity purification. Then, use the PageRuler™ Prestained Protein Ladder to determine the size of the purified protein to confirm that the purification was succussed. It can also be deduced from the gel image that the efficiency and necessity of XAC Purification via the reduction of non-specific binding protein shown from the lane of His-tag to XAC-affinity column purification.

The functionality of the purified receptors was validated by radioligand binding assay as shown in Figure 4 following.

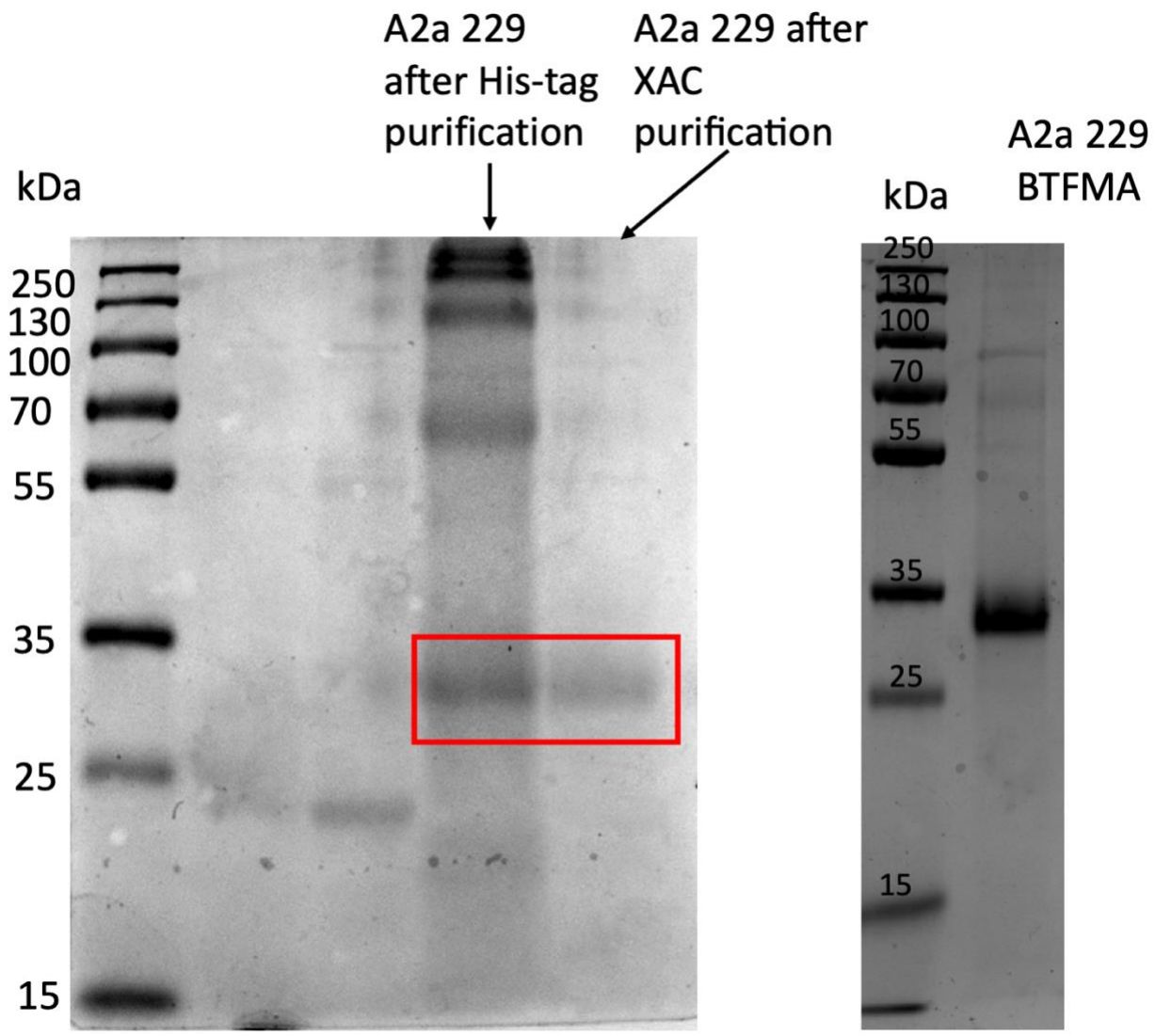


Figure 3. SDS-PAGE of expressed $A_{2A}R$ after His-tag and XAC affinity purification. SDS-PAGE was performed at 120 V for 90 mins with PageRuler™ Prestained Protein Ladder.

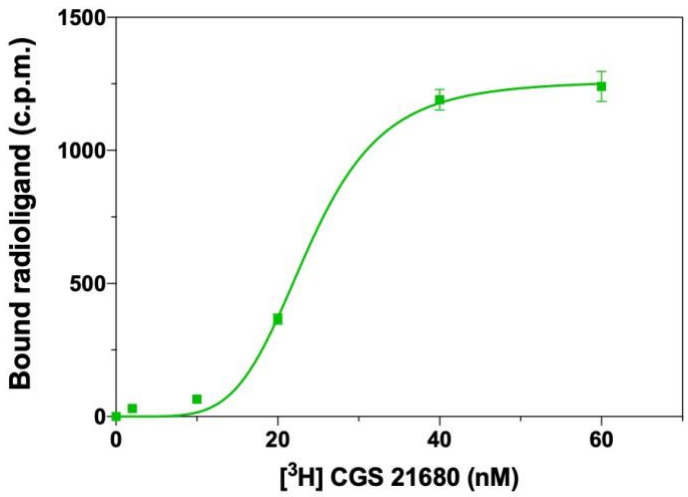
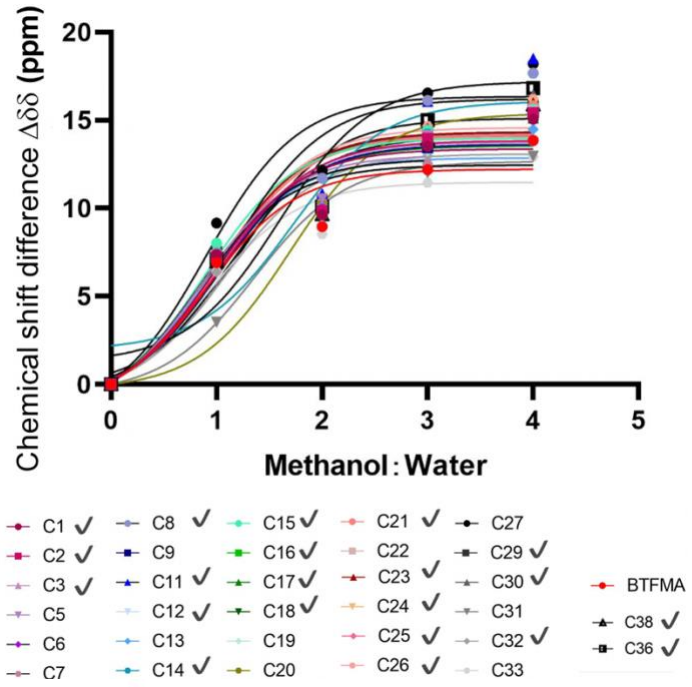


Figure 4. Radio ligand binding saturation assay results of $A_{2A}R$. Data processed by Excel and Prism.

As the method established, we went to evaluate each potential ^{19}F compound to determine their microenvironmental sensitivity as a function of solvent polarity described in section 1.2.2 before attachment of $A_{2A}R$_V229C. To evaluate the sensitivity of each potential tag to microenvironmental changes, a series of methanol/water mixtures were prepared with a range from (MeOH: H_2O = 4) to (MeOH: H_2O = 1/4) by creating a polarity screening system, mimicking micro-electrostatic environmental system. As shown in Figure 5, the red curve represents the BTFMA. The other compounds are named in an order and their chemical name, structures, and CAS number can be found in the Table 1. More than 10 compounds exhibited higher sensitivities to the surrounding environmental changes than the BTFMA, which provided us an enriched pool in the following experiments. Due to the time limits as a master's student and the difficulty of receptor preparation, the work is currently being performed by my colleagues.

A



B

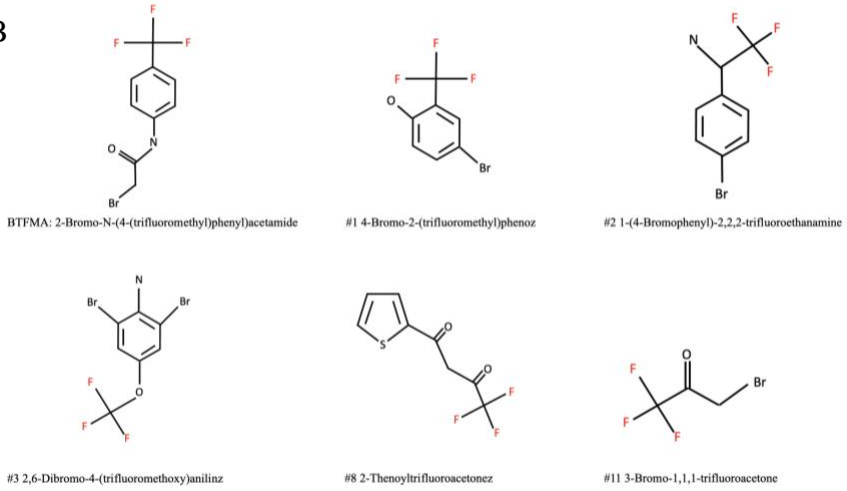


Figure 5. Free tag screening as a function of mixture of methanol : water.
A. Checked compounds are the ones showing the potential better than the BTFMA from the free tag perspective.
B. Compound structure with their names. (Continues on the next page)

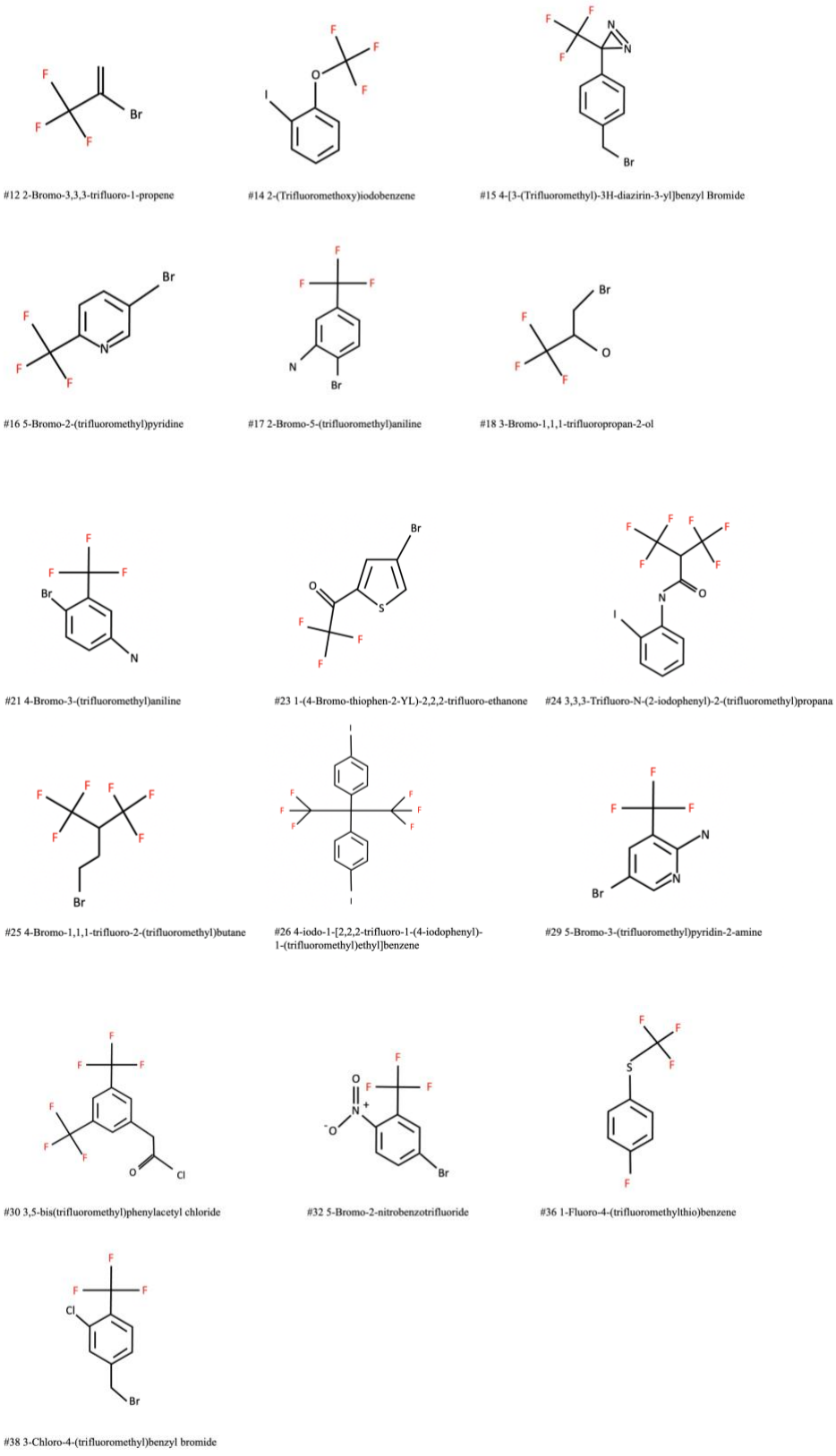


Figure 5. Free tag screening as a function of mixture of methanol : water.
B. Compound structure with their names. (Continued)

1.4 Summary

One of our goals is to obtain a ^{19}F probe better than the current BTFMA tag in probing the GPCR conformations, through the free tag screening and then receptor conjugation screening. Due to the time limits, the current research only allowed me to finish the first step of free tag screening using the solvent polarity as a solvent system, mimicking the microenvironmental changes. The results indicated more than ten ^{19}F compounds exhibited higher sensitivities than the BTFMA. It is worthy of note, a domain rotation indicator (TFKE) was developed during this screening process. [25]

Chapter Two: Preparation of Yeast-derived G proteins

2.1 Introduction

The G protein is also named guanine nucleotide-binding proteins which indicates their ability of binding to the nucleotides guanosine triphosphate (GTP) and the guanosine diphosphate (GDP). [17, 31] Some G proteins are small proteins that only have one single subunit, but the one associates with GPCRs are usually heterotrimeric which have three subunits: α subunit, β subunit, and γ subunit. The Gα and Gγ are the subunits attached to the plasma membrane by lipid anchors, while the Gβ usually form a complex with Gγ. [10] And Gα is classified into several families including $G\alpha_i$, $G\alpha_s$, $G\alpha_{12/13}$, and $G\alpha_q$. Different Gα can bind to different GPCRs, for example, $G\alpha_s$ can interact with the $A_{2A}R$. The Gα subunit only binds to either GTP or GDP at once which depends on which state G protein is: GTP binding when G protein is active while GDP binding indicates G protein inactive. The initial signal received from GPCR will pass to heterotrimeric G protein, resulting in conformational changes of G proteins and triggers G protein nucleotide exchanges. [25] The GDP attaches to the α subunit will be replaced by GTP and results in GTP-bound Gα and a Gβγ complex dissociates from GPCR. [25] This interaction leads to some activity like enzyme production, adenylyl cyclase for example, in the downstream signal transduction. The yeast system has its advantages in expressing G proteins for receptor conformation and dynamics study using NMR. First, it is easy for manipulation for isotope labeling compared to other expression systems with a superb GPCR production at are relatively low cost. [28, 34, 35] The *P. pastoris* expression system has shown promise in GPCR preparation, with many advantages that other eukaryotic expression systems cannot match. *P. pastoris* is simply to modify and high

productivity in protein expression and it is also known for its capability of protein production, especially complex proteins. On another side, the expression of GPCR in *P. pastoris* for structural study has already been conducted, so *P. pastoris* has been chosen for the expression of G protein here.

2.2 Materials and Methods

2.2.1 Plasmid Construction

Plasmid constructs and flowchart of expression are shown in Figure 6 and Figure 7. Gene was synthesized ahead and intergraded into the genome of *P. pastoris* by restriction enzymes: *Bam*HI and *Not*HI. The $A_{2A}R$ had a size of 35.1 kDa with a TEV protease cleavage site and α-Factor peptide ahead of it. It also had FLAG and His10 tags at the N- and C- terminal ends, respectively.

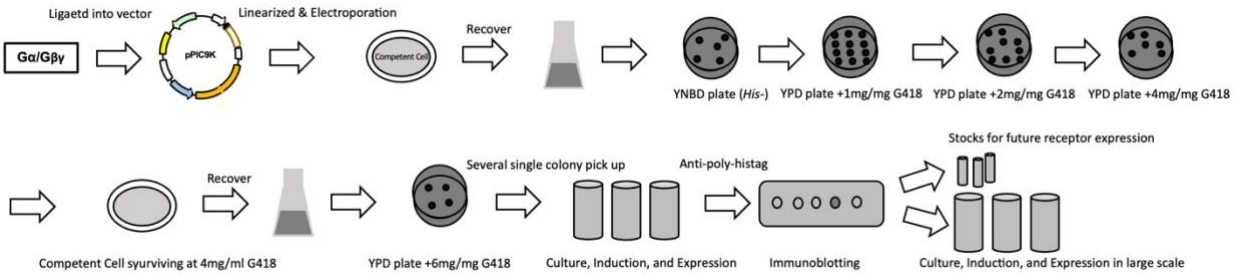


Figure 6: The general procedure for G protein prepration. The Gα and Gβγ preparation are slightly different, which are described in each section.

Compared to the $A_{2A}R$, Gα and Gβ had 6 × His, Gγ did not have 6 × His gene because it co-expressed with Gβ. All three G subunits had no FLAG tag or α-Factor peptide. Plasmid sconstructed by inserting target gene(s) into the vector, pPIC3K, using XL-10 Gold as competent cells. [27] The recombinant plasmids were digested via *Bam*HI-HF and *Not*I-HF restriction enzymes in NEB CutSmartBuffer at 37 °C for 2 h and then digested with Quick CIP at 37 °C for

30 min. The target genes including Gα (10,385 bp) and Gβ (10,447 bp) and Gγ (9,198 bp) contained *Bam*HI and *Not*I restriction sites at N- and C- termini respectively and synthesized based on the *P. pastoris* codon. [36] The codon-optimized genes were also treated with *Bam*HI-HF and *Not*I-HF restriction enzymes in NEB CutSmartBuffer at 37 °C for 2 h. The digested gene fragments and recombinant vector were confirmed by DNA electrophoresis and purified via QIAquick Gel Extraction Kit. Measure the concentration using NanoDrop ™ Lite

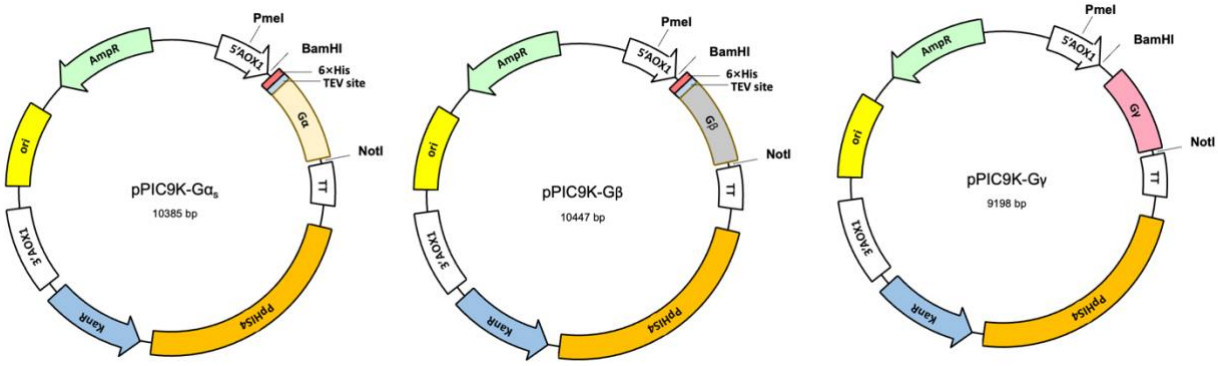


Figure 7: Reconstructed plasmids of Gα, Gβ and Gγ

spectrophotometer before ligating them using T4 DNA Ligase with NEB T4 DNA Ligase Buffer at room temperature (20 °C) for 2 h or 4 °C overnight. Ligation product stored at -20 °C if not immediate use.

2.2.2 DNA gel electrophoresis

Using the Bio-rad Agarose Powder make 1% DNA gel by completely dissolving it into TAE electrophoresis buffer (Thermo Fisher). Add ethidium bromide (EtBr) to a final concentration of approximately 0.5 μg/mL to allow the visualization of DNA under ultraviolet (UV) light. Pouring complete dissolved agarose into well with well combs to cool down. Put the solidified gel into the gel box and fill it with TAE electrophoresis buffer. Mix the sample with DNA 6 × gel loading dye

(Thermo Fisher) and load into well same as a molecular weight ladder. Run the gel at 70V for 20-40 mins and visualize the gel under UV lights.

2.2.3 G protein expression

Ligation products, constructed plasmid containing Gα or Gβγ, were amplified using XL-10 Gold competent cells. [37] The constructed plasmids were linearized by the *Pme*I enzyme in NEB CutSmart® Buffer at 37 °C for at least 2h. Two times volume ethanol was added to the linearized plasmid and incubate on ice for 5 min. Then centrifuged at 4 °C at 16,200 × g for 10 min and discarded the supernatant. The fume hood dries the linearized plasmid before added water to dissolve it. Around dissolved in distilled deionized water, 10 μg linearized plasmid was mixed with *P. pastoris competent cells* to perform electroporation under 1,500 V charging voltage, 25 μF capacitance, and 400 Ω resistance. Right after the electroporation, 1 mL of ice-cold 1 M sorbitol was added and incubated at 30 °C for 3 h without shaking. Spread the transformed cells on YNBD (1.34% yeast nitrogen-based w/o amino acid, 0.0004% D-Biotin, and 1% dextrose) plates. As shown in Figure 6, the colonies grown on the YNBD plates picked up and cultured on YPD plates with gradient G418 concentrations starting from 1 mg/mL G418 to 4 mg/mL for subsequent screening.[27]

A single colony derived stock spread on YPD (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose) plates containing 1mg/mL G418 was inoculate into 4 mL YPD medium and cultured at 30 °C overnight with shaking (275 rpm). Then, transferred into 200 mL BMGY (1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB (yeast nitrogen base) without amino acids, 0.00004% (w/v) glycerol and 0.1 M PB (phosphate buffer) pH 6.5) medium in 14 mL Falcon

tubes at 30 °C for at least 24 h with shaking (275 rpm) until the absorbance of $A_{595\ nm}$ in the range of 2 to 6. The medium was then transferred into 1L BMMY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB (yeast nitrogen base) without amino acids, 0.00004% (w/v) biotin, 0.1 M PB (phosphate buffer) pH 6.5, 0.04% (w/v) histidine, 3% (v/v) DMSO and 10 mM theophylline) at 20 °C for 60 h with shaking (275 rpm). Methanol (0/5% (v/v)) was added into the medium every 12 h to induce the Gα or Gβγ expression. [23, 27]

Similar to shown in Figure 2, the cell pellets were collected by centrifuge at 3,800 × g for 10 min at 4 °C in 400 mL centrifuge bottles and then washed with G protein washing buffer P1 (50 mM HEPES, pH 8.0). Then the cell pellets were resuspended into G Protein Lysis buffer P2 (50 mM HEPES, pH 8.0, 10% Glycerol, 100 mM NaCl) at a ratio of four times the volume of cell pellets itself. The cells in P2 were broken via Microfluidizer for four cycles on the ice at 15,000 psi and centrifuge at 9,720 × g for 30 min at 4 °C in 250 mL centrifuge bottles to get rid of cell debris. [27, 28]

2.2.4 Gα proteins purification

The supernatant bind to the pre-balanced Talon Resin for 2 h at 4 °C with. The amount of resin usage for a 6 g membrane should be around 2 mL. Then, the Talon Resin was packed onto a column and performed purification. First, using G protein washing buffer P1 washed on column 5 column volumes and then eluted the expressed protein, which supposed to be Gα (45.7 kDa), strategically recycling using 10 mL G protein elution buffer P3 (50 mM HEPES, pH 8.0 and 300 mM imidazole) at a gravity rate. [29] Use Ultra-15 Centrifuge filters 3K to concentrate the collected eluted protein to 2 mL and ready to load onto the FPLC system. The FPLC system was

balanced in advance with Q Buffer A – Gα (50 mM HEPES, pH 8.0, 50 μM GDP and 1mM $MgCl_2$), and the elution process was done by both Q Buffer A-Gβγ and Q Buffer B – Gα (50 mM HEPES, pH 8.0, 50 μM GDP, 1mM $MgCl_2$ and 1,000 mM NaCl) under the condition of flow rate: 1.0 mL/min; maximum column pressure: 0.5 MPa and fraction: 2.0 mL. Based on the result elution profile, the corresponding fractions were collected and concentrated. The BCA kit was used to identify the concentration of the Gα.

2.2.5 Gβγ proteins purification

The supernatant bind to the pre-balanced Talon Resin for 2 h at 4 °C with shaking. The amount of resin usage for a 6 g membrane was around 2 mL. Then, the Talon Resin was packed onto a column and performed purification. First, using G protein washing buffer P1 washed on column 5 column volumes and then eluted the expressed proteins, which was supposed to be Gβ (38.7 kDa) and Gγ (7.6 kDa), strategically recycling using 10 mL G protein elution buffer P3 (50 mM HEPES, pH 8.0 and 300 mM imidazole) at a gravity rate. [29] Use Ultra-15 Centrifuge filters 3K to concentrate the collected eluted receptors to 2 mL and ready to load onto the FPLC system. The FPLC system was balanced in advance with Q Buffer A – Gβγ (50 mM HEPES, pH 8.0 or 20 mM Bis-Tris, pH 6.5), and the elution process was done by both Q Buffer A-Gβγ and Q Buffer B – Gβγ (50 mM HEPES, pH 8.00 or 20 mM Bis-Tris, pH 6.5 and 1,000 mM NaCl) under the condition of flow rate: 1.0 mL/min; maximum column pressure: 0.5 MPa and fraction: 2.0 mL. Based on the result elution profile, the corresponding fractions were collected and concentrated. The BCA kit was used to determine the concentration of Gβγ.

2.2.6 SDS-PAGE and Western-Blotting

Preparing the SDS-PAGE gel based on the size of protein and our choice was 12% for separating gel. The purified protein mixture to the 2 × loading (50 mL receipt: 1 mg bromophenol blue, 12.5 mL 1.5 M Tris-HCl, pH 6.8, 2.5 mL 10% SDS (sodium dodecyl sulfate) and 5 mL 100% glycerol) at 1:1 ratio. Load the sample onto the gel with PageRuler™ Prestained Protein Ladder and run at 120 V for 90 min. The SDS-Page running buffer was prepared as 25 mM Tris-base, 192 mM glycine, and 0.01 % SDS. Fixed the gel in 50% (v/v) methanol and 10% (v/v) glacial acetic acid for 5-10 min and then stained the gel in 50% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.1% (v/v) Coomassie brilliant blue R-250 for at least 30 mins. Destained the gel with 40% (v/v) methanol and 10% (v/v) glacial acetic acid and then visualized the gel using Bio-rad ChemiDoc system.

The Western-blotting gel was run side by side with SDS-Page gel in order to have identical localization of protein bands on gels. Prepare the PVDF (polyvinylidene fluoride) membrane by cutting it into the same size as the filter paper and larger than the SDS-Page gel. Presoak the PVDF into the methanol for 2 min then submerge the PVDF in ice-cold Western blot transfer buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) for 5 min. After running the gel at 120 V for 90 min, assemble the gel and PVDF membrane like a sandwich using filter paper and a sponge as the bread. Run blot at 100 V for 90 min or 300 mA for 75 min and make sure the negative side of the machine matches the gel side. The time and voltage can be slightly optimized base on the condition until the protein is adder is fully transferred. Submerge membrane in blocking buffer (125 mM NaCl, 25 mM Tris base, pH 7.5, 0.3% Tween-,20 and 3% non-fat milk) for 1 h at room temperature with shaking and then discard the blocking buffer. Apply antibody incubation buffer (Anti-His/Anti-

Flag antibody diluted to 1:2000 with blocking buffer) at room temperature with shaking for 1 h and collect the antibody incubation buffer and store at -20 °C for reuse. Wash the PVDF membrane with washing buffer (125 mM NaCl, 25 mM Tris base, pH 7.5, 0.3% Tween-20) for 5 min at room temperature with shaking followed by distilled water twice. The membrane was finally visualized by Super Signal West Pico Plus Chemiluminescent Substrate (usually 6 mL in total should be enough for one membrane) after shaking at room temperature for 5 min.

2.3 Result and Discussion

2.3.1 Gα proteins purification

The Gα subunit was around 45 kDa which fits the expectations. Both the left gel on Figure 8: His-tag fraction, and the right gel: concentrated ones, did have the intense band around that size. The FPLC curve in the middle showed the elution program and resultant elution profile for the Gα.

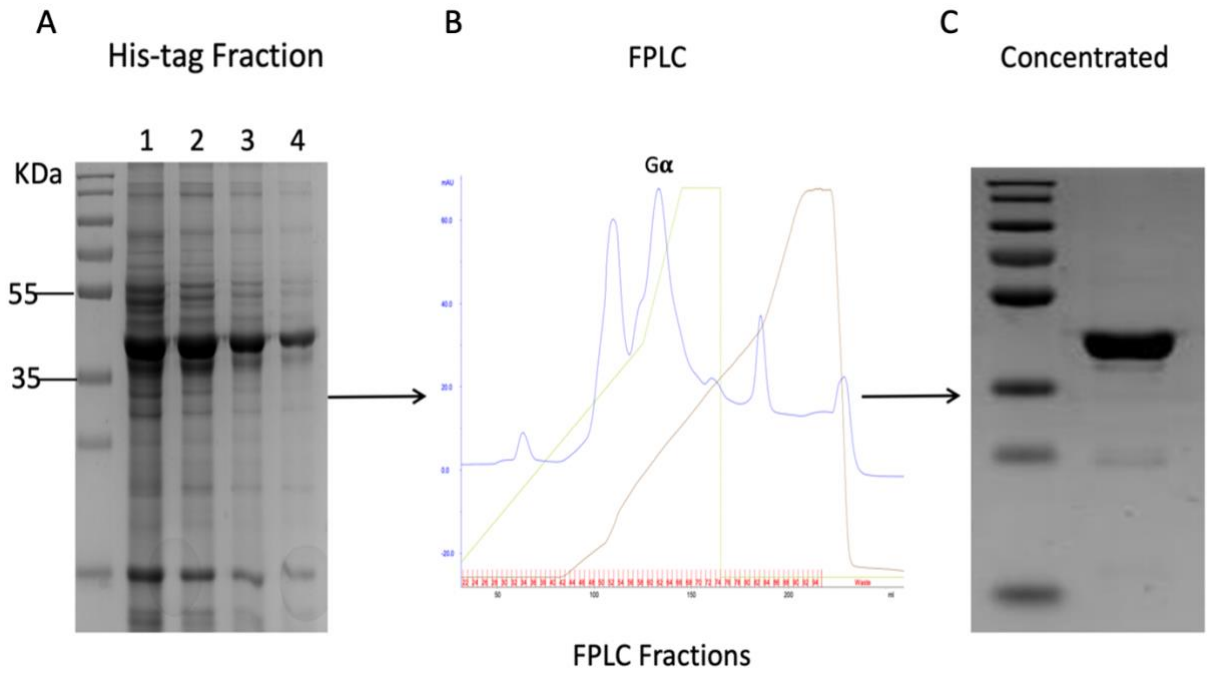


Figure 8. Purification results shown in FPLC curves with gel confirmations of yeast-derived Gα
A. His tag purification of yeast-derived Gα show in SDS-PAGE
B. FPLC resulting curve for the Gα
C. Collected FPLC fractions based on the result curve. Yeast-derived Gα shown in SDS-PAGE.

2.3.2 Gβγ proteins purification

The Gβ subunit should be slightly below the 40 KDa band on the ladder while the complex of **G**βγ **should be around 45 kDa which fits between the 40 kDa and 50 kDa bands in the ladder lane.** In Figure 9, the FPLC curves presented for Gβγ and the SDS Page showed the band stands for the concentrated fractions from FPLC. The two peaks in the FPLC result curve also indicates there are both Gβ and Gβγ complex showing up in the purified protein.

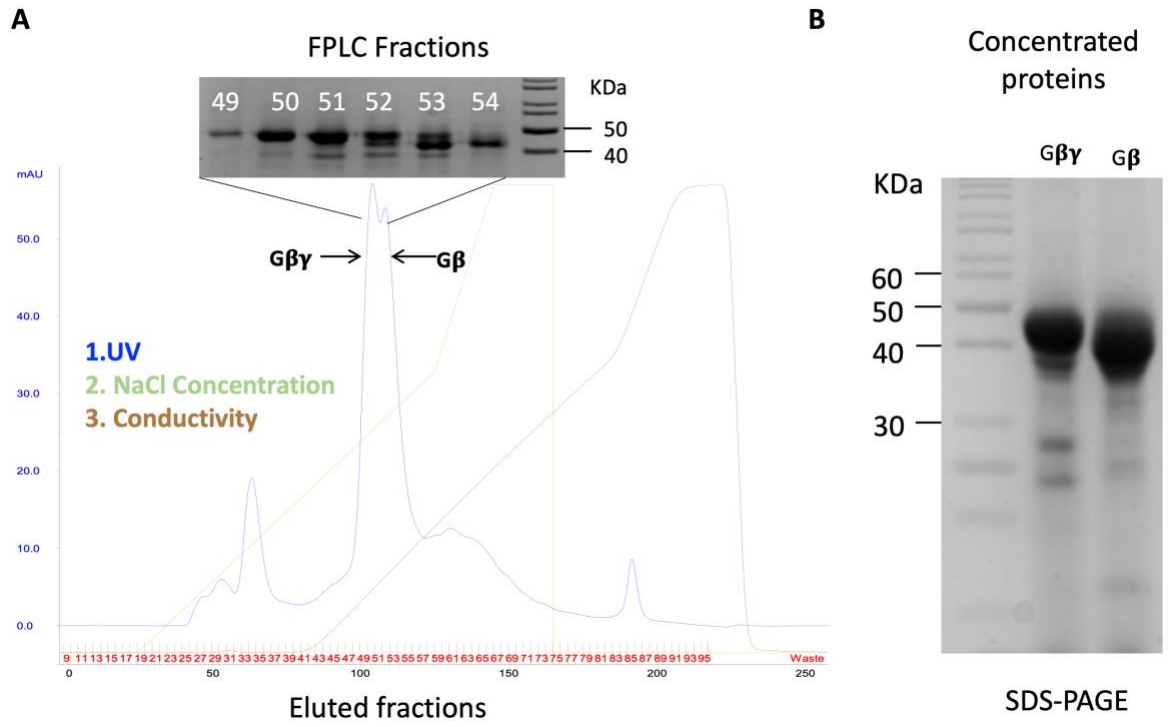


Figure 9. Purification results shown in FPLC curves with gel confirmations of yeast-derived Gβγ

A. FPLC resulting curve for Gβγ

B. Purification of yeast-derived Gβγ shown in SDS-PAGE

2.4 Summary

This project enabled us to establish two protocols in preparing yeast-based G proteins, including Gα and Gβγ, though their functionality still needs to be further validated using various tools such as observation of radioligand bindings of the receptor when they engage the receptors.

Conclusion

One of the goals of this thesis is to obtain a desirable ^{19}F chemical tag better than the BTFMA in probing the GPCR conformational states and dynamics, which is partially accomplished here. Due to the limitation of time, the subsequent work in this direction is ongoing by other lab members.

As part of the effort for this thesis, we also successfully established platforms of expressing Gα and Gβγ using the yeast-based expression system, which will have significance to investigate the conformational transitions and dynamics of these G proteins by isotopically labeling each of G proteins while interacting with the receptors along with associated nucleotide exchanges.

Publications

1. Wang, X., Zhao, W., et al., *Trifluorinated Keto–Enol Tautomeric Switch in Probing Domain Rotation of a G Protein-Coupled Receptor.* Bioconjugate Chemistry, 2021. **32**(1): p. 99-105.
2. Zhao, W., X. Wang, and L. Ye, *Expression and Purification of Yeast-derived GPCR, Gα and Gβγ Subunits for Structural and Dynamic Studies.* Bio-protocol, 2021. **11**(4): p. e3919.

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Appendix A: Supplementary Tables

Table A1: Names and CAS numbers for the Trifluorinated Compounds Used in This Study

	Names	**CAS**
0	2-Bromo-N-(4-(trifluoromethyl)phenyl)acetamide	3823-19-6
1	4-Bromo-2-(trifluoromethyl)phenoz	50824-04-9
2	1-(4-Bromophenyl)-2,2,2-trifluoroethanamine	843608-46-8
3	2,6-Dibromo-4-(trifluoromethoxy)anilinz	88149-49-9
4	4-Bromo-N-(2,2,2-trifluoro-1-phenylethyl)benzamide	R269077
5	1,1-Dibromo-3,3,3-trifluoroacetone	431-67-4
6	4-Amino-3-iodobenzotrifluoride	163444-17-5
7	Potassium (bromomethyl)trifluoroborate	888711-44-2
8	2-Thenoyltrifluoroacetonez	326-91-0
9	2-Amino-5-bromobenzotrifluozide	445-02-3
11	3-Bromo-1,1,1-trifluoroacetone	431-35-6
12	2-Bromo-3,3,3-trifluoro-1-propene	1514-82-5
13	4-Bromo-3-nitrobenzotrifluoride	349-03-1
14	2-(Trifluoromethoxy)iodobenzene	175278-00-9
15	4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]benzyl Bromide	92367-11-8
16	5-Bromo-2-(trifluoromethyl)pyridine	436799-32-5
17	2-Bromo-5-(trifluoromethyl)aniline	454-79-5

Table A1(Continued)

18	3-Bromo-1,1,1-trifluoropropan-2-ol	431-34-5
19	4'-Bromo-2,2,2-trifluoroacetophenone	16184-89-7
20	3-Bromo-1-(2,2,2-trifluoroethyl)pyrazole	1354706-17-4
21	4-Bromo-3-(trifluoromethyl)aniline	393-36-2
22	1-Iodo-3-(trifluoromethyl)benzene	401-81-0
23	1-(4-Bromo-thiophen-2-YL)-2,2,2-trifluoro-ethanone	1252046-14-2
24	3,3,3-Trifluoro-N-(2-iodophenyl)-2-(trifluoromethyl)propanamide	
25	4-Bromo-1,1,1-trifluoro-2-(trifluoromethyl)butane	203303-02-0
26	4-iodo-1-[2,2,2-trifluoro-1-(4-iodophenyl)-1-(trifluoromethyl)ethyl]benzene	55100-57-7
27	1-(4-Bromophenyl)-4,4,4-trifluorobutane	18931-61-8
28	Potassium 4-bromophenyltrifluoroborate	374564-35-9
29	5-Bromo-3-(trifluoromethyl)pyridin-2-amine	79456-34-1
30	3,5-bis(trifluoromethyl)phenylacetyl chloride	174083-39-7
31	4-Fluoro-3-(trifluoromethyl)benzyl bromide	184970-26-1
32	5-Bromo-2-nitrobenzotrifluoride	344-38-7
33	2-Amino-3-bromo-5-(trifluoromethyl)pyridine	79456-30-7
34	3-Chloro-4-iodobenzotrifluoride	141738-80-9
35	Potassium trifluoro(iodomethyl)borate	888711-47-5
36	1-Fluoro-4-(trifluoromethylthio)benzene	940-76-1
37	4-Ethoxy-3-(trifluoromethyl)benzyl bromide	1206593-30-7
38	3-Chloro-4-(trifluoromethyl)benzyl bromide	361393-92-2
40	4-IodophenylsμLfur Pentafluoride	286947-68-0