ABSTRACT

INVESTIGATION OF GAI1 PROTEIN HOMODIMERIZATION IN LIVE CELLS USING FÖRSTER RESONANCE ENERGY TRANSFER (FRET) AND BIMOLECULAR FLUORESCENCE COMPLEMENTATION ASSAY (BIFC))

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The classical GPCR signaling pathway, where a heterotrimeric G protein-GPCR interaction is sufficient to transmit the signal to effector proteins has been replaced by a heteromeric G protein-GPCR homo- or hetero-dimer interaction model over the past two decades. These studies demonstrate that GPCRs that interact with each other couple with a heteromeric G protein. In recent years, evidence suggests that dimer of GPCR dimers is required for some complex signal transductions. In these studies, it was proposed that this heteromeric tetramer formed by the dimerization of the dimers brought two G proteins close enough to each other for protein-protein interaction. It is not clear if GPCR tetramerization is required for G-protein dimerization or dimerization can occur independent of the GPCRs. On the other hand, studies on small G-proteins (Ras family), which are structural homologs of G alpha subunits of heteromeric G-proteins, shows that dimerization can be independent of the receptors and necessary for various signaling pathways.

Within the scope of this study, Gα protein homodimerizations were qualitatively and quantitatively investigated in live cells using Bimolecular Fluorescent

Complementation Assay (BiFC) and Förster resonance energy transfer (FRET) method. To achieve this, the Gαi1 protein gene was labeled from various positions, including (G60-Y61, L91-K92 and A121-E122), with Enhance Green Fluorescent Protein (EGFP) which was derived from *Aequorea victoria* and mCherry fluorescent protein which is a monomeric derivative of DsRed. In addition, the Gαi1 gene was labeled with split EGFP parts which are N-terminus EGFP and C-terminus EGFP for the Bimolecular Fluorescence Complementation Assay. All labeled proteins were co-transfected into *Mus musculus* Neuroblastoma-2a (N2a) cells and interactions were imaged using spinning disc confocal microscope and analyzed.

The findings of this study could help us understand the molecular mechanisms required for Gα dimerization and the dynamics of these proteins. Also, GPCR interactions with various effectors during complex signal transductions and the requirement of these receptors during Gα dimerizations can be studied with the techniques optimized in this study.

Keywords: G-protein, GNAI1, Homodimerization, Förster Resonance Energy Transfer (FRET), Bimolecular Fluorescence Complementation Assay (BiFC)

ÖZ

Gαi1 PROTEİN HOMODİMERİZASYONUN CANLI HÜCRELERDE FÖRSTER REZONANS ENERJİ TRANSFERİ (FRET) VE BİMOLEKÜLER FLORESAN TAMAMLAMA (BiFC) METOTLARI KULLANILARAK İNCELENMESİ

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Heterotrimerik G protein-GPKR etkileşiminin, sinyali efektör proteinlere iletmek için yeterli olduğu klasik GPKR sinyal yolağı, son yirmi yılda bir heterotrimerik G protein-GPKR homo- veya hetero-dimer etkileşim modeli ile değişmiştir. Bu çalışmalar, birbirleriyle veya diğer GPKR'ler ile etkileşime giren GPKR'lerin heteromerik G proteinler ile etkileşime girdiğini göstermektedir. Son yıllarda ki araştırmalar, bazı karmaşık sinyal yolakları için GPKR dimerlerinin, dimerleşmesinin gerekli olduğunu göstermektedir. Bu çalışmalar, dimerlerin dimerizasyonu ile oluşan hetero tetramerlerin, iki G proteinini, protein-protein etkileşimi için yeterli yakınlığa getirdiğini öne sürmektedir. GPKR'lerden bağımsız olarak G-protein dimerizasyonu veya dimerizasyon için GPCR tetramerizasyonunun gerekli olup olmadığı bilinmemektedir. Öte yandan, heterotrimerik G-proteinlerinin Gα alt ünitelerinin yapısal homologları olan küçük G-proteinler (Ras ailesi) üzerinde yapılan çalışmalar, dimerizasyonun reseptörlerden bağımsız olabileceğini ve çeşitli sinyal yolları için gerekli olduğunu göstermiştir.

Bu çalışma kapsamında Gα proteinlerinin homodimerizasyonu, canlı hücrelerde, Bimoleküler Floresan Tamamlama testi (BiFC) ve Förster Rezonans Enerji Transferi (FRET) kullanılarak nitel ve nicel olarak araştırılmıştır. Bunu başarmak için, Gαi1 protein geni, Gαi1 geninin içinden (G60-Y61, L91-K92 ve A121-E122), *Aequorea victoria'* dan elde edilen yeşil floresan geni ve monomerik DsRed geninin türevi olan mCherry floresan proteinleri ile etiketlenmiştir. Bunun yanı sıra, Gαi1 geni, bimoleküler floresan tamamlama testi için, N-terminal EGFP ve C-terminal EGFP olarak adlandırılan bölünmüş EGFP parçaları ile etiketlenmiştir. *Mus musculus* Neuroblastoma-2a (N2a) hücrelerine eş transfekte edilen tüm etiketli proteinler ve bu proteinlerin etkileşimleri konfokal floresan mikroskop ile analiz edilmiştir.

Bu çalışmanın bulguları, Gαi dimerizasyonu için gerekli moleküler mekanizmaları ve bu proteinlerin dinamiklerini anlamamıza yardımcı olacaktır. Ayrıca, karmaşık sinyal iletimi sırasında çeşitli efektörlerle GPKR etkileşimleri ve Gαi dimerizasyonları sırasında bu reseptörlerin gereksinimi, bu çalışmada optimize edilmiş tekniklerle incelenebilir.

Anahtar Kelimeler: G-Protein, GNAI1, Homodimerizasyon, Förster Rezonans Enerji Transfer (FRET), Bimoleküler Floresan Tamamlama Metodu (BiFC)

To my dad

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ABBREVIATIONS

A2AR	Adenosine A2A Receptor
AC	Adenylyl Cyclase
ADP	Adenosine Diphosphate
AGE	Agarose gel electrophoresis
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
bp	base pair
BiFC	Bimolecular Fluorescence Complementation
BSA	Bovine Serum Albumin
cAMP	cyclic AMP
$CaCl_2$	Calcium chloride
CFP	Cyan Fluorescent Protein
cDNA	Complementary Deoxyribonucleic Acid
DAG	Diacylglycerol
D2R	Dopamine D2 Receptor
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetraacetic acid

ECL1	Extracellular loops
Bp	base pair
GAP	GTPase activating protein
GDP/GTP	Guanine di/tri- phosphate
ICL1	Intracellular loops
Kb	kilo base pair
LB	Luria Bertani
mCherry	Monomeric Cherry
mEGFP	Monomeric Enhanced Green Fluorescent Protein
PDE	Phosphodiesterase
PI-PLC	Phosphoinositide-specific phospholipase C
PPIs	Protein-protein interactions
SBE	Spectral bleed-through

CHAPTER 1

INTRODUCTION

1.1. GTP-binding Proteins

1.1.1. Classification of Heterotrimeric G Proteins

In a multicellular organism, continuance of homeostasis depends on the processing of information through complex networks by the cells. Moreover, organisms can response to many dynamic conditions. In order to achive these processes, intracellular or extracellular signals must be expanded, transduced and conclusively converted to proper physiological responses. These molecular events are classified as signal transduction in organisms. Guanine nucleotide-binding proteins (G proteins), that is an evolutionarily highly conserved group of molecules, are fundamental for signal transduction (Tsvetanova, Irannejad, & von Zastrow, 2015). Signal transduction begins with binding of a ligand, which could be hormones, neurotransmitters, ions, glycoproteins or sensory molecules such as light or taste molecules, to their relevant receptors on the cell membrane. G-protein coupled receptors (GPCRs) are the primary and most abundant cell surface receptors (Vilardaga, Agnati, Fuxe, & Ciruela, 2010). GPCRs signal mainly through their intimate functional and physical coupling among classes of guanine di/tri- phosphate (GDP/GTP) nucleotide-binding proteins (G proteins).

G proteins play a central role in signal transduction, including cell growth, protein synthesis, membrane vesicle transport. G proteins are classified as small G proteins (small GTPases) and heterotrimeric G proteins in mammalian cells. Small G proteins are required to sustain cell polarity, cell cycle progression, cytoskeletal reorganization in eukaryotic cells. Although the small G protein family extends over 150 members at present, they are mainly subdivided into five groups including Ras, Rho, Arf/Sar,

Rab and Ran according to their sequence, function, and structure. Among these members, Ras is the most predominant member of small G proteins in cells (Song *et al.*, 2019). Besides the small G proteins, heterotrimeric G proteins were first recognized and described by Alfred Gilman and Martin Rodbell 30 years ago. Heterotrimeric G proteins influence numerous effector proteins such as ion channels, adenylyl cyclase, phosphoinositide-specific phospholipase C (PI-PLC) and phosphodiesterase (PDE) (Zachariou, Duman, & Nestler, 2012). They are comprised of three distinct subunits which are named as alpha (α), beta (β) and gamma (γ). The β and γ subunits found as Gβγ complex in the cell. G proteins are named as their Gα subunits such as Gαi, Gαs, Gαq and Gα12/13.

In human genome, there are 16 Gα genes that encode 23 known Gα proteins. Gα subunits range in size from 39 to 45 kilodaltons (kDa) and they share between 35 to 95% sequence identity. These proteins can be categorized into four major classes according to their sequence similarity: Gα (s/olf), Gα (i1/i2/i3/o/t-rod/t-cone/gust/z), Gα (q/11/14/16) and Gα (12/13) (McCudden, Hains, Kimple, Siderovski, & Willard, 2005). There are two members in Gαs family: Gαs and Gαolf. Gαs is expressed in most of the cell types and Gαolf is particularly observed in the olfactory sensory neurons. Gαi family is the most diverse and abundant group of Gα proteins. They are displayed in most cell types.Gαq family composed of Gαq, Gα11, Gα14, and Gα16. Gαq and Gα11 are ubiquitously expressed, while Gα14 and Gα15/16 expression are more limited in some cell types. Gα14 is mainly found in the lung, kidney and liver, and Gα15/16 is specifically expressed in hematopoietic cells. Gα12 family, which are Gα12 and Gα13, are displayed in most cell types (Syrovatkina, Alegre, Dey, & Huang, 2016) (Figure 1.1A).

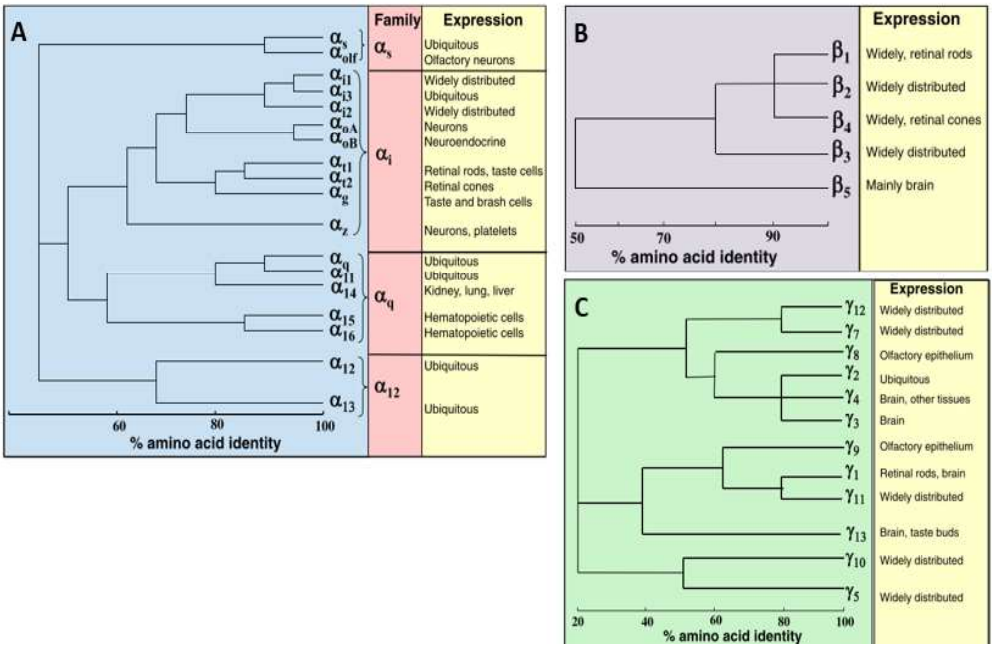


Figure 1.1: Sequence identity of the human and mouse Gα, Gβ and Gγ subunits and their expressions**.** (Taken from Syrovatkina *et al.*, 2016).

Heterotrimeric G-proteins also contain the attached Gβγ subunit in addition to Gα subunits. 5 Gβ subunits (Gβ1, Gβ2, Gβ3, Gβ4, and Gβ5) found in human and mouse genomes. Sequence similarities of Gβ1, Gβ2, Gβ3, and Gβ4 are between 80 to 90%. On the other hand, Gβ5 has only 50% sequence similarity to other Gβ subunits. While Gβ5 is mostly displayed in brain cells, other Gβ subunits are found in most cell types (Figure 1.1B). In addition to Gβ subunits, there are 12 Gγ genes in the mouse and human genomes. They are relatively more diverse and share similarities, between 30 to 80% sequence identity (Figure 1.1C).

1.1.2. Membrane Localization and Structure of Heterotrimeric G Proteins

The crystal structure of Gα, Gβγ and Gαβγ have been resolved (Lodowski, Pitcher, Capel, Lefkowitz, & Tesmer, 2003). The structure of Gα subunit composed of two domains which are α-helical domain and a nucleotide-binding domain with high structural homology to Ras-family GTPases (Ras-like domain) (McCudden *et al.*, 2005). The α-helical domain is unique among the heterotrimeric G proteins, whereas the nucleotide-binding domain (Ras-like) is shared in all members of the GTPase superfamily (Liu, Clark, Sharma, & Northup, 1998) (Figure 1.2 A). The α-helical domain includes six α-helices that create a lid over the nucleotide-binding domain, which buries the bound nucleotide in the center of the protein. Variation of the helical domain is greater than the Ras-like domain among Gα families according to the comparison of the amino acid sequences (Simon, Strathmann, & Gautam, 1991). These findings verify that helical domains are a determinant of the selectivity of interaction between Gα and G-protein coupled receptors (Liu *et al*., 1998). Various functions of the helical domains have been discussed, including enhancement to the affinity of GTP binding, participating in effector coupling, acting as a tethered intrinsic GTPase activating protein (GAP) and transition of the active-inactive conformation of Gα protein (Codina & Birnbaumer, 1994; Markby, Onrust, & Bourne, 1993). The GTPase domain consists of a six-stranded β-sheet that enclosed by five α-helices (Lambright, Noel, Hamm, & Sigler, 1994). Most highly conserved sequences found on the five loops that contain the guanine nucleotide-binding sequences, the Mg_2^+-binding domain, the diphosphate-binding (P-) loop, and guanine ring-binding motifs are located in the GTPase domain. This domain also includes three flexible regions represented as switch-I, -II and -III that modify the structure in response to GTP binding and hydrolysis (Mixon *et al.*, 1995) (Figure 1.2 A).

The structure of the Gβ subunit is composed of seven-bladed β-propeller with seven WD40 sequence repeats. Each blade consists of four antiparallel β-strand where one WD40 presents the last three strands in one blade. The last blade needs the N-terminus

of Gβ for the contribution of the fourth β-strand (Sondek, Bohm, Lambright, Hamm, & Sigler, 1996). The remaining N-terminal residues utilize an α-helical structure that created a coiled-coil which is necessary for interaction with Gγ subunit (Garritsen, van Galen, & Simonds, 1993) (Figure 1.2 B). This interaction is important for the proper folding and function of Gβ subunits (Dupré, Robitaille, Rebois, & Hébert, 2009). Members of the Gγ family are formed from two α-helices linked by a loop. The C terminal helix interacts with the blade 5 and 6, while N-terminal helix interplays with the N-terminus of Gβ subunits (Sondek *et al.*, 1996).

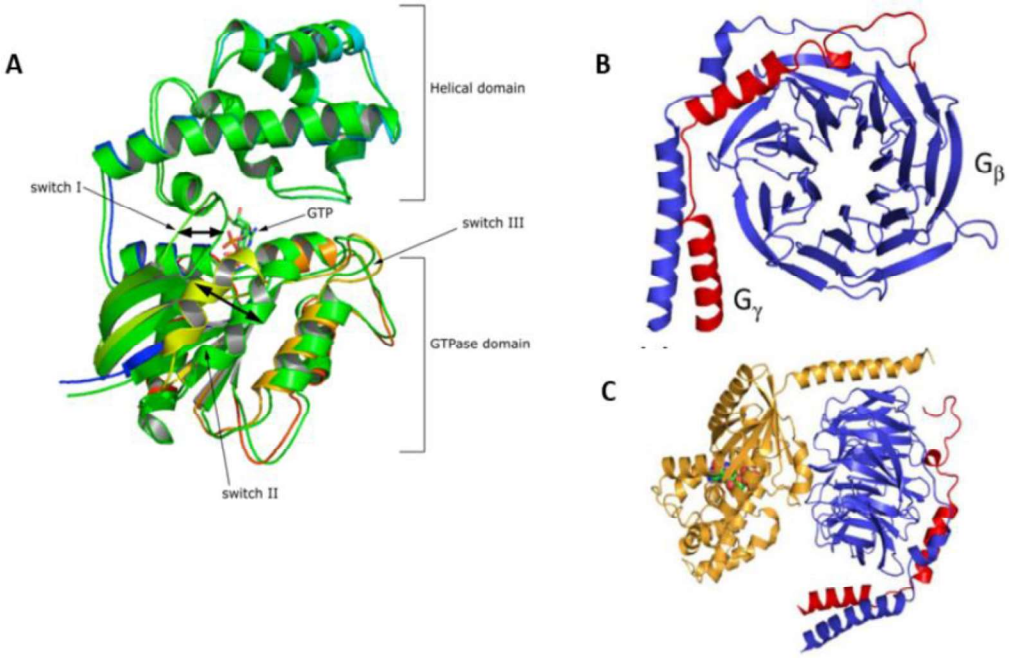


Figure 1.2: Structural features of heterotrimeric G protein subunits. (A) The crystal structure of Gα illustrates the GTPase (Ras-like) domain, the helical domain, and the flexible regions; switch regions I, II and III. (B) Gβ1γ2 dimer, where blue is Gβ1 and red is Gγ2. (C) Gαi1β1γ2 heterotrimer, where yellow is Gαi1, blue is Gβ1 and red is Gγ2 (Taken from Hermans M. J. W, 2008 & Syrovatkina, Alegre, Dey, & Huang).

1.1.3. G Protein Signaling Pathway

Communication is necessary between cells in multicellular organisms as this provides coordination of activities for individual cells. This communication is provided by

extracellular stimuli, including neurotransmitters, hormones, interleukins or differentiation, and growth factors. These stimuli are identified by receptors that are found on the cell membrane of responding cells. Signal transduction is a process that includes; receiving the stimuli from the outside of the cell, evaluation of the signal and translation to convenient response in the cells of organisms. This response could include cell growth, cell death, cell division, metabolism, cell migration, or immune/neuronal communication. Receptors that detect the messenger from the outside of the cell include G-protein coupled receptors, protein-tyrosine kinase receptor, ion channels, or serine/threonine kinase receptor (van der Geer, 2013).

The G-protein signaling pathway depends on activation/inactivation cycle of GPCRs (Kristiansen, 2004). The classical view of the G-protein signaling pathway describes the connection between G-protein and receptor and this association occurs only after the receptor activation by ligands (Manglik & Kruse, 2017). Although different classes of GPCRs have various ligand binding sites, all conformational changes by activation of receptors occur with in the 7-TM domains (Park, Lodowski, & Palczewski, 2008) (Figure 1.3A). Heterotrimeric G protein consists of guanine nucleotide (GDP) bound Gα and Gβγ subunits which linked to Gα subunit (P. Zhang, Kofron, & Mende, 2015). Activation of receptor results in conformational changes and this promotes the release of GDP from Gα and subsequent binding of GTP which is found in excess amounts in the cells compared to GDP (McCudden *et al.*, 2005). GTP binding to Gα subunit reduces its association to Gβγ (Lambert, 2008). Both Gβγ and GTP-bound Gα are able to interact with downstream effectors. Termination of the signal depends on the intrinsic guanosine triphosphatase (GTPases) activity of Gα that hydrolyze GTP to GDP, resulting in the conversion of Gα to its inactive state. The inactive state of Gα subunit triggers the reassociation of Gβγ to Gα that terminates all effector interactions (Ford *et al.*, 1998; Li *et al*., 1998) (Figure 1.3B).

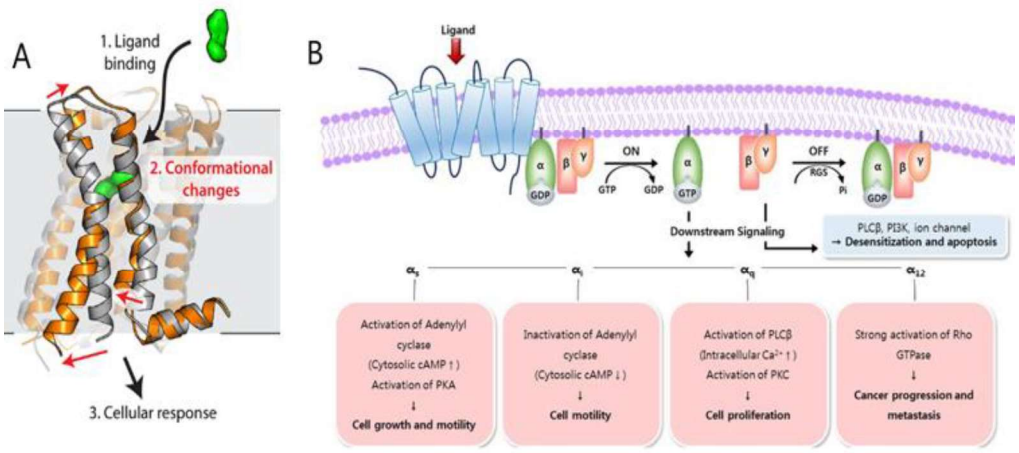


Figure 1.3: Illustration of G-protein signaling**.** A) Conformational changes in 7TM of GPCRs resulted from ligand binding. B) Classical view of the G-protein signaling pathway with GPCRs. Ligand binding of GPCRs triggers the activation of G-protein. GDP displaces with GTP and GTP binding catalyzes dissociation of Gβγ from Gα. Depending on the subclasses of Gα (Gαi, Gαs, Gαq and Gα12/13) various downstream effector molecules, such as protein kinase A, phospholipase Cβ (PLCβ), Rho GTPases and adenylyl cyclase are involved in physiology of cells (Adopted from (Jo & Jung, 2016; Manglik & Kruse, 2017).

1.1.4. G Protein Signaling Pathway Through Gαi and Gαs Proteins

Gαi and Gαs are the members of Gα family proteins. Gαi family is the largest and most diverse member of Gα family including Gαi1, Gαi2, Gαi3, Gαt, Gαg, Gαo, and Gαz. Gαi members expressed in many types of cells. Gαt is observed mostly in the rod cells and cone cells in the eye. Gαg couple with the taste receptor and Gαz is found in platelets and neuronal tissues (Wong *et al.*, 2000). Finally, Gαo is expressed mainly in the neuron. In addition, there are two members of Gαs protein: Gαs and Gαolf. While Gαs is observed in most cell types, Gαolf is found in only olfactory sensory neurons (Syrovatkina *et al*., 2016). Gαi and Gαs proteins exert their effect on

signal transduction by inhibitory and stimulatory effect on adenylyl cyclase, respectively. According to their effects, Gαi is named as inhibitory G protein and Gαs is called as stimulatory G protein. Upon activation of the receptor by a ligand, GDP is exchanged for GTP, following the dissociation of Gαi or Gαs subunit from Gβγ subunit. Figure 1.4 illustrates the cyclic functions of Gαi and Gαs proteins. The Gαs-GTP complex activates the adenylate cyclase which is an enzyme involved in the production of cyclic AMP (cAMP) (Bhagavan & Ha, 2011). It produces cAMP by generating a cyclic phosphodiester bond with the α-phosphate group of ATP. The substrate of adenylyl cyclase is a complex of ATP and Mg_2^+. As a result of this, Gαs-GTP complex is involved in the stimulation of the production of cAMP in cells. cAMP can be distributed through the cytoplasm, where it associates with various targets. Therefore, it is administered as a second messenger within the cells (Oliveira *et al*., 2010; Taskén & Aandahl, 2004). Protein kinase A (PKA) is the most common downstream target of cAMP. The PKA molecules release two catalytic subunits which have enzymatic activity in the target cells upon binding of four molecules of cAMP (Skalhegg & Tasken, 2000). Similar events are observed by binding a ligand that activates the Gαi and forming of Gαi-GTP complex. However, Gαi-GTP prevents activation of adenylate cyclase and generation of cAMP in cells (Duman & Nestler, 1999).

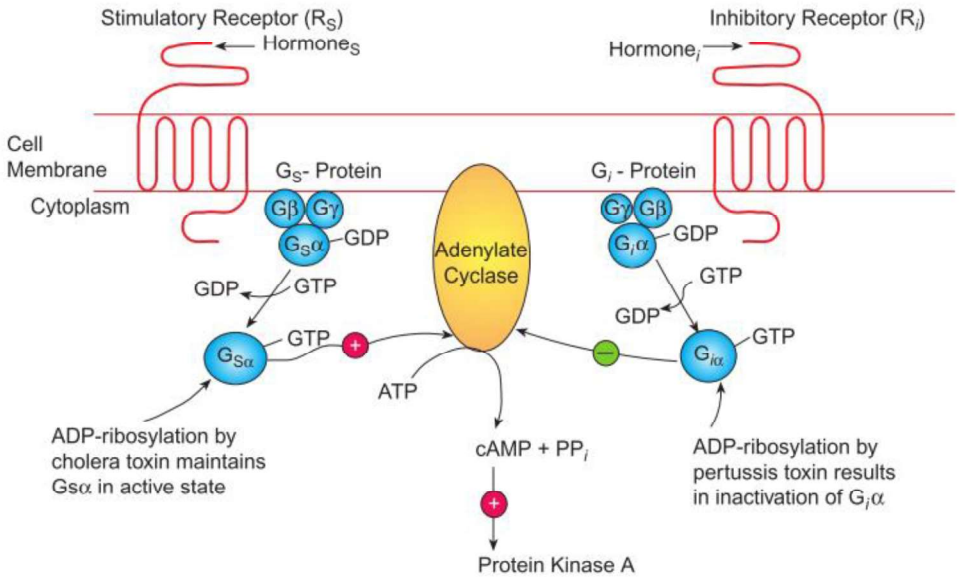


Figure 1.4: Illustration of signal transduction of Gαi and Gαs protein**.** Production of adenylate cyclase is inhibited by Gi and activated by Gs protein. Adenylate cyclase produces a second messenger that is cAMP from ATP. Then cAMP activates the downstream effector; PKA. Cholera and pertussis are regulatory toxins that inactive of Gαs and Gαi activation respectively.(Taken from Bhagavan & Ha, 2011).

1.2. G-Protein Coupled Receptors (GPCRs)

Cell signaling is an essential process needed for cell survival and cell growth. The basic cell signaling events involve a receptor that receives the signal outside of the cell or surface of other cells. The signal transduced via cell membrane receptors to effector molecules (Trewavas & Malho, 1997). The targets of these signals could be an intracellular receptor, transcription factors or various enzymes (Hucho & Buchner, 1997). One of the most important and diverse signaling cascades is initiated by coupling of Guanine nucleotide-binding protein (G-protein) with G-protein coupled receptors (GPCRs) (Temple & Jones, 2007). GPCRs have a significant impact on cellular functions. They operate important functions including smell, vision, senses of

taste and controlling a myriad of intracellular signals (Rosenbaum, Rasmussen, & Kobilka, 2009). It is thought that approximately 800 GPCR genes are found in the human genome (Gurevich & Gurevich,2017). As GPCRs play critical roles in many functions, defects in these receptors can be linked to various diseases including obesity, inflammation, hypertension, and blindness (Sensoy, Almeida, Shabbir, Moreira, & Morra, 2017).

Members of the superfamily of GPCRs share common structural features. They are composed of seven transmembrane α-helices (TM1-7) which are linked by three intracellular loops (ICL1, ICL2, and ICL3), and three extracellular loops (ECL1, ECL2, and ECL3) in each side of the cell membrane (Moreira, 2014). In addition, the amino terminus which is found outside of membrane can be involved in ligand binding, whereas the carboxy-terminus (C-terminus) located in the cytoplasm of cell interfere the interaction with heterotrimeric G proteins (van der Geer, 2013) (Figure 1.5)

Classification and nomenclature of GPCRs depend on their phylogenetic analysis of amino acid sequences, analysis of globular domains and motifs and their native ligands (Dong, Filipeanu, Duvernay, & Wu, 2007). Human GPCRs can be described into five separate groups of receptors which are rhodopsin, glutamate, adhesion, secretin, and frizzed-taste-2 based on their phylogenetic investigation (Kristiansen, 2004). The general activation process of GPCRs with their cognate G protein was already described under the topic of the G protein signaling pathway. After the GPCRs activation and signal transduction process through the G protein, GPCRs typically experience desensitization and internalization that are occupied by phosphorylation of the agonist-induced receptor by G-protein coupled receptor kinases (GRKs). Phosphorylation by GRKs promotes the recruitment of β-arrestin which terminates the G-protein mediated signaling. β-arrestin then targets the receptor to endocytosis via its direct interaction with clathrin and the adapter protein AP-2 (Cattaneo *et al.,* 2014).

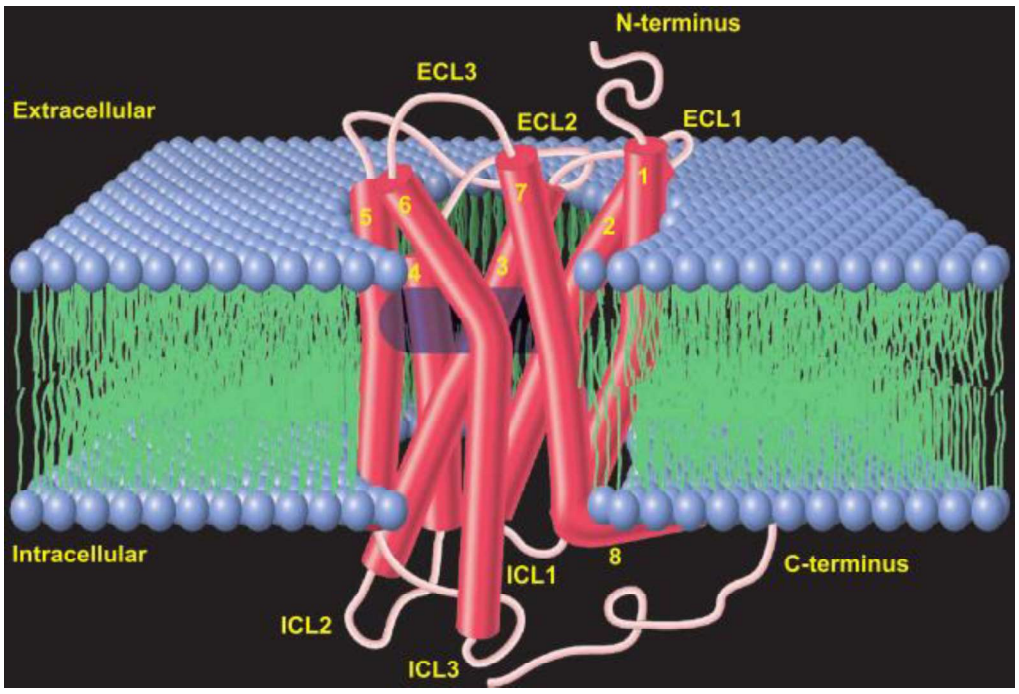


Figure 1.5: Schematic representation of the three-dimensional arrangement of the 7 transmembrane helices of a GPCR in the cell membrane (Taken from Kristiansen, 2004).

1.3. Homodimerization and Heterodimerization of Proteins

In biological systems, proteins can interact with other proteins to achieve complex functions including gene expression, cell-to-cell adhesion, communication, the activity of ion channels and enzymes (Inanobe & Kurachi, 2014). These interactions provided by the association of the same or different proteins to form supramolecular complex classified as protein oligomers. The oligomerization process is modulated by temperature, ligand or other proteins. Besides, protein oligomerization arises through covalent or weak bond interaction thus could be irreversible and/or reversible respectively. Oligomeric proteins consist of same and multiple subunits called homo-oligomeric protein or different subunits called hetero-oligomeric (Ali & Imperiali, 2005). Dimers and tetramers are more common states of homo-oligomers which is approximately four times more frequently observed in nature than hetero-oligomers (Kumari & Yadav, 2019).

Protein dimerization is common in G-protein coupled receptor family. Heterodimers in GPCRs have been widely investigated in co-transfected cells using pharmacological, biochemical and biophysical approaches (Derouiche & Massotte, 2018). Dimerization may play a significant role in regulating the functions of GPCRs and gives the novel functional entities to receptors. The disruption of receptor dimerization has a negative effect on the function of GPCRs (Bai, 2004). For instance, dimerization of the class C GPCRs such as GABA receptors is required for their function. In addition to GABA receptors, dimerization can be observed between Adenosine 2A (A2A) and Dopamine 2 (D2R) receptors which are classified as class A receptors. They are expressed at high concentration in the striatum. They are involved in motor coordination in the striatal pathway (Prezeau *et al*., 2010). The association of A2-D2 simultaneously takes place in the presence of ligands. Activation of the A2A receptor negatively modulates the D2 ligand binding affinity. Therefore, this allosteric modulation ruptures dopamine D2 receptor-dependent G protein signaling. Reciprocally, activation of D2 receptors by agonists negatively cross-modulate A2 receptor-ligand attachment and signaling (Derouiche & Massotte, 2018). In the heterodimerization of GPCRs, two different heterotrimeric G proteins can pair to heteromers, the overall complex constitutes a functional structure. In the case of D2 and A2 receptor heteromers, they can interact with both Gαi and Gαs protein respectively. Recently, computational and experimental studies demonstrate that receptor heteromer models brought two different Gα interacting with homo-dimers close to each other at the interaction distance (Navarro *et al*., 2016). These studies suggest that Gα proteins bound to receptor homodimers to interact with each other as a result of physical convergence during heterotetramer formation. Although the G proteins dimerization has newly become more of an issue, Ras protein family including N-Ras, K-Ras ve H-Ras which are structurally related with Gα protein are known to form dimers since 1988 (Chung, Lee, Lam, & Groves, 2016; Nan *et al*., 2015; Santos, 2014). Dimerization affects the functionality of Ras proteins. For instance, Raf kinase which is a downstream effector of Ras-guanosine triphosphate (GTP) (Muratcioglu *et al*., 2015) is only activated by dimerization of Ras proteins. In

addition, recent research shows that Ras dimerization has an inhibitory effect on tumor formation *in vivo* (Khan, Spencer-Smith, & O'Bryan, 2019).

1.4 Protein-Protein Interaction Detection Methods

The fate of proteins in a cell includes synthesis, maturation, vesicle budding, trafficking and degradation which are accomplished by heterogeneous protein-protein interactions (PPIs) (Xing, Wallmeroth, Berendzen, & Grefen, 2016). Because of the important roles of PPIs in these functions, it is becoming a major field of systems biology to study these interactions (Rao, Srinivas, Sujini, & Kumar, 2014). In order to predict the function of target proteins and the drug ability of molecules, protein-protein interactions have substantial effect (De Las Rivas & Fontanillo, 2010). Based on the different functional and structural properties of proteins, the classification of PPIs could be temporary or stable, homo- or hetero-oligomeric, obligate or non-obligate (Pan, Lahiri, Rajendiran, & Shanmugham, 2016). Detection of PPIs can be classified into two groups: high-throughput (Yeast two-hybrid and affinity purification) and low-throughput techniques (nuclear magnetic resonance spectroscopy, fluorescence resonance energy transfer, atomic force microscopy, and electron microscopy) *in vivo* and *in vitro* (Peng, Wang, Peng, Wu, & Pan, 2017).

1.4.1 Förster Resonance Energy Transfer Method (FRET)

Recently, the tendency in the application and development of fluorescent tools to find answers to a wide variety of scientific and technological problems (Zhang *et al.*, 2019) in biology, chemistry, and physics increased. The fluorescence resonance energy transfer method has been broadly used in biological studies to examine the roles, dynamics, properties, and interaction of molecules including nucleic acids and proteins (Okamoto & Sako, 2017). Förster or Fluorescence Resonance Energy Transfer method (FRET) was firstly identified by Theodore Försters in 1948 with the goal of understanding the efficiency of photosynthesis (Lemke, Deniz, & Groarke, 2016). FRET is a non-radiative energy transfer process including an excited state

fluorophore called donor that transfers the energy to a ground state fluorophore called as acceptor molecules (Lemke *et al.*, 2016). Energy transfer in FRET is the result of long-range dipole-dipole interactions within donor and acceptor. It depends particularly on the distance of acceptor and donor ranging from 40-100 Å (Chen & Yu, 2019). The occurrence of the FRET depends on some basic condition of the donor-acceptor pair called a FRET pair. First of all, the donor's emission spectrum partly should overlap with the excitation spectrum of the acceptor molecule. Secondly, the distance between donor and acceptor molecules should be larger than the collision diameter. Finally, the dipoles should not position perpendicular to one another for the FRET pair (Chen & Yu, 2019).

According to Förster theory, the energy transfer rate $k_T(r)$ from a donor to acceptor is calculated with the equation:

$$k_T(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6$$

where r is the distance between acceptor and donor, R_0 is the Förster distance or radius and τ_D is the fluorescent decay rate of the donor in the absence of acceptor. Therefore, FRET efficiency can be described as:

$$E_{FRET} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

Förster principle has a significant advantage that the donor-acceptor separation distance R can be concluded from observation of E(FRET). This provides the use of

FRET as a nanoscale ruler for the molecular systems effectively (Wallace & Atzberger, 2017).

1.4.2 Bimolecular Fluorescence Complementation Assay (BiFC)

Most of the cellular functions are achieved and managed by a complex interaction of proteins. It has been suggested that the functions of the cells are achieved by nearly 130.000 protein interactions in the human body (Venkatesan *et al.,* 2009). The visualization of protein complexes in living cells is significant for the determination of interactions in the regular cellular environment and for the classification of subcellular positions of protein complexes (Kerppola, 2008). Recently, bimolecular fluorescence complementation (BiFC) assay has been extensively used for the determination and identification of protein-protein interactions (PPIs) (Miller, Kim, Huh, & Park, 2015). This technique provides the visualization of the protein interactions and alterations in organisms and living cells (Kerppola, 2009). BiFC was first defined by Hu *et al.* (2002) using the enhanced yellow fluorescent protein that is an Aequorea victoria GFP variant protein (Lai & Chiang, 2013). BiFC depends on the association between two non-fluorescent fragments of a fluorescent protein able to produce a fluorescent complex. This association is facilitated by the interaction between target proteins that fused to the non-fluorescent fragments of fluorescence protein (Figure 1.6) (Vidi, Ejendal, Przybyla, & Watts, 2011).

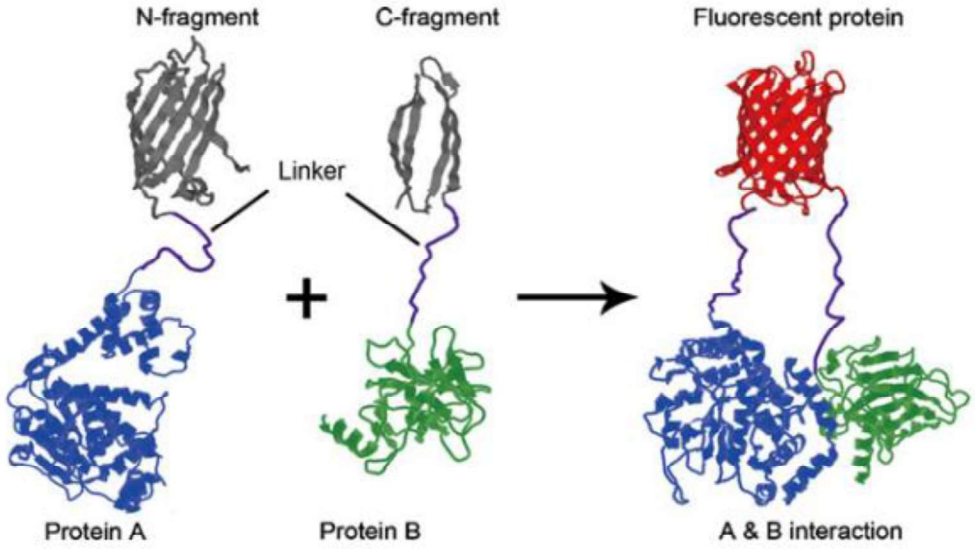


Figure 1.6: Schematic diagram of BiFC. Proteins of interest (Protein A and protein B) fused to N- and C- terminal fragments of fluorescent protein respectively. The fusion protein of A-N and B-C does not emit fluorescence under excitation. When protein A and B interact, N- and C- fragments of fluorescent protein are brought together, the fluorescent characteristic is reconstructed and emission of fluorescence under excitation is reproduced (taken from Fan *et al.*, 2008).

The BiFC signal can be detected by fluorescent microscope or examined by flow cytometry without any other treatment to cells. One of the advantages of BiFC is the direct visualization of temporal and spatial interaction between protein complexes in live cells with minimum perturbation (Miller *et al.,* 2015). Another is fluorescence signal is readable even at low expression level of target proteins (Morell, Espargaro, Aviles, & Ventura, 2008). BiFC assay also can be easily adapted for different cellular conditions and the procedure is comparatively simple. Although the BiFC method is relativly easy and it has advantages among the PPIs detection methods, it has also some limitations. One of these limitations is nonspecific interaction increases when fusion protein overexpressed (Vidi *et al.*, 2011). Another one is the slow maturation time of fluorescent proteins. Thşs makes it inappropriate for real-time observation of

transient interaction that disappear before BiFC detection (Hu, Chinenov, & Kerppola, 2002).

1.5 Aim of the Study

Protein dimerization is common in G-protein coupled receptor family. Heterodimers in GPCRs have been widely studied in co-transfected cells using pharmacological, biochemical and biophysical approaches (Derouiche & Massotte, 2018). Dimerization has a significant role in regulating the functions of GPCRs and their interacting proteins. This gives novel functional roles to GPCRs and to interacting G-proteins. During the heterodimerization of GPCRs, two different heterotrimeric G proteins can pair to heteromers, the overall complex constitutes a functional structure.

In these days, experimental and computational studies demonstrate that receptor heteromer models brought two different Gα protein, interacting with homo-dimers of GPCRs, close to each other at the interaction distance (Navarro *et al*., 2016). These studies suggest that Gα proteins bound to receptor homodimers, could interact with each other as a result of physical convergence during heterotetramer formation.

The aim of this study is qualitatively and quantitatively investigate Gα protein homodimerization in live cells using Bimolecular Fluorescent Complementation Assay (BiFC) and Förster resonance energy transfer (FRET) method. To achieve this, the Gαi1 protein gene, which is a member of G-protein family, was tagged with Enhance Green Fluorescent Protein (EGFP) and mCherry fluorescent protein genes. In addition, the Gαi1 gene was cloned with split EGFP parts which were N-terminus EGFP and C-terminus EGFP for the Bimolecular Fluorescence Complementation Assay. All tagged protein genes were co-transfected to N2a cells with various combinations and dimerizations were analyzed both qualitatively and quantitatively using confocal fluorescence microscope images.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Mouse Neuroblastoma Neuro2a (N2a) Cell Line and Cell Media

Mouse Neuroblastoma cell line Neuro 2a (N2a) was chosen for the visualization of fluorescently labeled Gα proteins and protein-protein interactions. This cell line is suitable for the transfection process. They are easily cultivated and proliferated and can be differentiated into neurons within a few days. N2a cells were supplied from ATCC company as CCL-131 *Mus musculus* brain neuroblastoma, ENGLAND.

Growth medium of N2a cell line includes OptiMEM®I, 44.5% Reduced Serum Medium with L-glutamine (Invitrogen, Cat#31985047), Dulbecco's Modified Eagle Medium DMEM, high glucose with L-glutamine (Invitrogen, Cat#41966029), 10% Fetal Bovine Serum (Biological industries, Cat#04127-1B) and 1% Penicillin/Streptomycin solution (Biological industries, Cat# 03-031-1B). Opti-MEM and DMEM were filtered by using Millipore Stericup® Filter Unit. Cells were incubated in Nuve® EC 160 CO_2 incubator at 37 °C with 5 % CO_2. Cell culture studies (passages, transfection, etc.) were performed in Nuve® LN 120 laminar flow cabinet. Cells were passaged every 3 days when they reach approximately 80-90% confluency. Phosphate Buffered Saline (PBS) solution was applied for washing the dead cells and waste materials. The trypsin-like solution which is TrypLE™ Express with Phenol Red (Invitrogen, Cat#12605-028) was used to lift the cells from the T25 flask. Formulation of DMEM and PBS presented in Appendix A.

2.1.2. Other Chemicals and Materials

All chemicals which were used in this thesis studies supplied from Sigma Chemical Company (NY, USA) and Applichem (Darmstadt, Germany). DNA polymerases, T4 DNA ligases, GeneRuler 1 kb (#SM0311) and GeneRuler 1 kb plus (#SM0313) DNA markers were purchased from Thermo Scientific (MA, USA). GeneJET PCR purification kit (#K0702) and Plasmid Miniprep Kit (#K0503) were supplied from Thermo Scientific (MA, USA). The gel extraction kit was obtained from QIAGEN (Cat#28704) (Düsseldorf, Germany). For the transfection method, Lipofectamine® LTX and Plus™ Reagent by Invitrogen (CA, ABD) was used as a reagent. Restriction enzymes were from New England Biolabs (MA, USA). T-25 cell culture flasks were obtained from Stastedt (Nümbrecht, Germany) and the glass-bottom dish (35 mm) was from In vitro Scientific (CA, USA). The functionality test for Gαi and Gαs which is cAMP-Glo assay (#V1502) was obtained from Promega (WI, USA).

Live cell imaging of Gα was performed by Leica Microsystems CMS GmBH's DMI4000B confocal microscope with Andor DSD2 spinning differential disc confocal device. PixFRET a plug-in for ImageJ program was used for FRET analysis of the images.

2.1.3. Bacterial Strain and Bacterial Culture Media

Escherichia coli XL1 strain was used for bacterial transformation. The strain was grown in liquid and solidified form of Luria Bertani (LB) solution. The components of LB (Appendix A) solution were dissolved in distilled water and sterilized at 121 °C for 20 minutes by use of Nüve OT 40L autoclave. Required antibiotics which are ampicillin (100 µg/mL) or kanamycin (50 mg/mL) were used for selection. Bacterial colonies were grown in solidified LB and incubated at 37 °C for 16 hours in an incubator ZHWY-200B by Zhicheng Instruments. Inoculated colonies were grown in liquid LB solution in the rotary shaker ZHWY-200B by Zhicheng Instruments at 200 rpm for 16 hours at 37 °C.

2.1.4. Plasmids, Primers, and Sequencing

GNAI1 and GNAS plasmids were obtained from Addgene. cDNA of mCherry fluorescence protein in pCS2-mCherry vector (Accession Number: ACO48282) and cDNA of mEGFP in pEGFP-N1 vector (Accession Number: AAB02574) were gifted by Prof. Dr. Henry Lester, California Institute of Technology (CA, USA). pcDNA 3.1 (-) (mammalian expression vector) with a CMV promoter (Appendix B) was gifted by Prof. Dr. Ayşe Elif Erson Bensan, Middle East Technical University (Ankara, TURKEY).

Primers were purchased from Integrated DNA Technologies (IDT) (IO, USA) and PRZ Biotech (Ankara, TURKEY). G protein genes that are labeled with fluorescence protein gene were sequenced by Molecular Cloning Laboratories (MCLAB) (CA, USA).

2.2. Methods

2.2.1. Preparation of Competent *E. coli* Cells by Rubidium Chloride Method

XL1 blue strains of *Escherichia coli* cells were utilized for the preparation of competent cells. A single colony from XL1 blue strain on the LB plate was inoculated into 2.5 ml of LB medium without antibiotics. Inoculation was performed at a shaker incubator for 12-16 hours at 37 °C and at 200 rpm. On the following day, the suspension was subcultured in a 1:100 ratio to LB medium. 20 mM $MgSO_4$ was added into the subculture. The colony was grown until the optical density reaches 0.4-0.6 at 600 nm (nearly took 2-3 hours). After the desired density was reached, the mixture was centrifugated at 4000 rpm at 5 min at 4 °C. The supernatant was discarded. Pellet was resuspended in 20 ml TBFI and incubated on ice for 5 min. After incubation, the cells were centrifugated at 4000 rpm for 5 min at 4 °C. The supernatant was removed and the pellet was resuspended with 2 ml of TBFII solution.

Subsequently, cells were incubated on ice for 45 min then aliquoted 50 µl /Eppendorf and quickly frozen in liquid nitrogen. Aliquots were stored at -80 °C for further usage. The preparation of TFBI and TFBII was summarized in Appendix A.

2.2.2. Polymerase Chain Reaction (PCR)

In this study, polymerase chain reaction was applied for the amplification of fluorescence proteins such as mEGFP, mCherry and split EGFP (N-EGFP &C-EGFP) with approximately 24 bp overhangs, matching to the integration site of target Gα proteins with the 18 bp linker (TCTGGAGGAGGAGGATCT). 5' forward and 5' reverse primers were designed to contain both of the overhang regions mattching target G proteins and 18 bp long linker. Designed primers listed in Appendix E. Optimal PCR conditions shown in Table 2.1.

Table 2.1 Optimal PCR conditions.

Reagent	**Amount**
5X Phire Reaction Buffer	10 µl
Phire HS II DNA Polymerase	1 µl
Template DNA (Fluorescent protein gene)	250-300 ng
10 Mm dNTPs	1 µl
Forward primer	1.25 µl (20 pmol)
Reverse primer	1.25 µl (20 pmol)
DMSO	11.5 µl (3%)
Nuclease free water	Up to 50 µl
total	50 µl

Pre-denaturation	98 °C	30 sec	
Denaturation	98 °C	10 sec	34 cycle
Annealing	58 °C	30 sec	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	

2.2.3. PCR Integration Method (Overlap Extension PCR Method)

In this study, mEGFP, mCherry and split EGFP (N-EGFP and C-EGFP) genes were inserted between G60-Y61, A121-E122, and L91-K92 positions in Gαi gene and between E73-S85 and R154-S155 positions in Gαs gene. In the PCR integration method, two consecutive PCR reactions occur. In the first PCR reaction, fluorescence

protein genes (mEGFP, mCherry, split EGFP) were amplified with the overhangs which were compatible with the sequences of the G proteins that they were intended to be labeled. In the second PCR, first PCR products were used as primers and G protein genes were used as a template for the second PCR reaction (Figure 2.1). The optimal template to primer ratio is 1:5 in the PCR integration method. Integration PCR conditions are in Table 2.2.

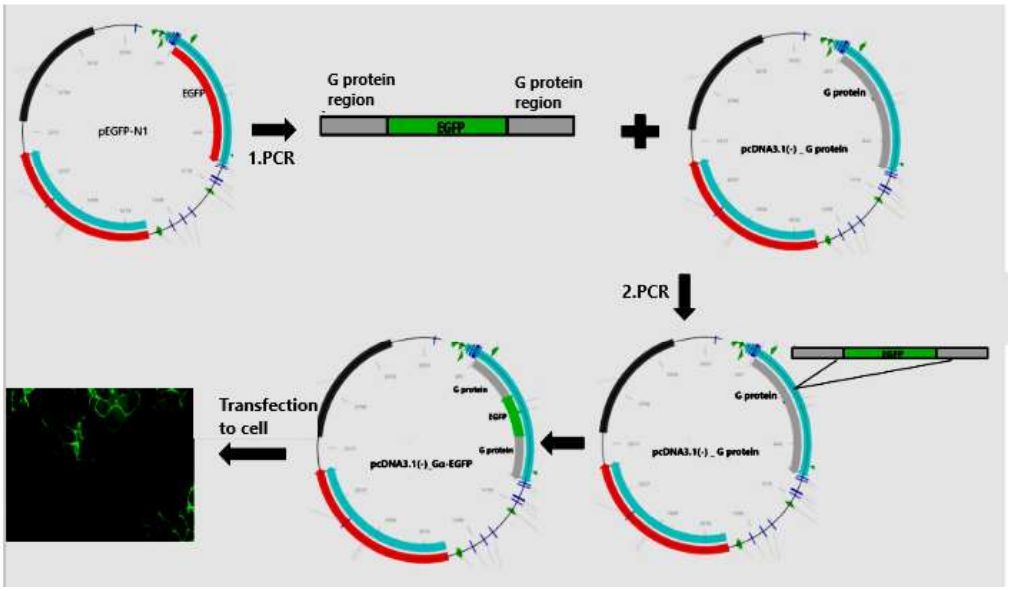


Figure 2.1: Demonstration of PCR integration method.

Table 2.2: Optimal integration PCR conditions

Reagent	Amount
Phire Green Hot start II PCR Master Mix	25 µl
Template (Gαi1 in pcDNA 3.1 -)	100 ng
1st PCR products	500 ng
DMSO	1.5 µl (3%)
Nuclease free water	Up to 50 µl
total	50 µl

Pre-denaturation	98 °C	3 min	
Denaturation	98 °C	30 sec	18 cycle
Annealing	51-65 °C	1 min	
Extension	68 °C	2 min/kb	
Final extension	68 °C	5 min	

2.2.4. Agarose Gel Electrophoresis

Products of PCR were controlled with agarose gel electrophoresis according to their sizes. Agarose gel was prepared as a 1% ratio. Agarose powder was dissolved in 1X TAE buffer by using the microwave oven. After cooling, ethidium bromide (EtBr) was added to observe the DNA under the UV light. The mixture of agarose and EtBr was then poured to electrophoresis tray. 6X DNA loading dye (Thermo Scientific, #R0611) was added to PCR samples. Afterward, the dye-sample mix and GeneRuler 1 kb DNA Ladders (Thermo Scientific, #SM0311) were loaded into the wells. The gel was run at 90-120 V for 40-45 min in the 1X TAE buffer. Preparation of the 1X TAE buffer can be found in Appendix A.

2.2.5. DNA Extraction from Agarose Gel

Amplification and size of PCR products were controlled after the agarose gel electrophoresis. Confirmed samples of DNA extracted from agarose gel by using QIAGEN® Gel Extraction Kit (Cat# 28704). The protocol of QIAGEN® Gel Extraction Kit was followed. In the last step of extraction, to elute DNA, 30 µl of nuclease-free water was added instead of 50 µl of buffer EB that was provided with the kit.

2.2.6. DNA Amount Determination

The BioDrop µLITE spectrophotometer was used for the quantification of the DNA amount. 1 µl of the sample solution was loaded onto the spectrophotometer and the measurement was done as directed by the user's manual.

2.2.7. Restriction Enzyme Digestion

Restriction enzymes were supplied from New England Biolabs Inc. (NEB). Restriction enzymes digestion was performed for obtaining sticky ends in the targeted genes. According to NEB's instructions, 1 unit of enzymes required to digest 1 µg of DNA in a 50 µl volume. In this study, approximately 200-300 ng of DNA was restricted with 0.25 µl of each enzyme with 1.5 µl CutSmart® NEB buffer supplied with the enzymes. The final volume was completed to 20 µl with nuclease-free water. DNA and enzyme mixtures then were incubated at 37 °C for 2 hours.

2.2.8. PCR Purification

PCR purification was applied to remove the components of restriction enzymes digestion and PCR purification. PCR purification was performed by using the Thermo Scientific GeneJet Purification kit (#K0702). The procedure was carried out according to the instruction manual of the kit. To elute the DNA, 30 µl of nuclease-free water was rather than 50 µl of buffer EB in the last step of the protocol.

2.2.9. Ligation

Ligation is a process that covalently connect the desired genes into a compatible digested vector backbone. The ligation involves DNA fragments that have sticky ends generated by restriction enzymes digestion. In this study, ligation reactions were performed in 1:5, 1:10 and 1:20 vector: insert ratio. For the reactions, 1 µl of T4 DNA ligase enzyme (NEB, Cat#0202T), 2 µl 1X T4 DNA ligase buffer added to the mixture

and the final volume was completed to 20 µl with nuclease-free water. The mixture was then incubated at room temperature for 3 hours.

2.2.10. Transformation of Competent *E. Coli* Cells

The bacterial transformation was applied for transferring the vector containing the desired gene to host bacteria cells. In this study, the XL1 blue strain of *E.coli* cells was used as competent cells. Cells from -80 °C freezer chilled on ice for 15 min. 5 µl of ligation product was added to cells. The mixture of DNA-competent cells was incubated on ice for 30 min. After incubation, cells were heat-shocked at 42 °C for 45 sec. Afterward, the cells were incubated on ice for 5 min. In the following step, liquid LB media was added to cells till the total volume becomes 1000 µl. Cells then were shaken at 37 °C, 200 rpm for 1 h. At the end of the transformation, 150 µl of cell suspension was spread to the LB agar plate prepared with the required antibiotics. Agar plates were incubated at 37 °C for 14-16 hours for growing colonies.

2.2.11. Plasmid Isolation from E. Coli

The isolation of plasmid DNA was performed by using Thermo Scientific's GeneJET plasmid miniprep kit (#K0503). A single colony was inoculated into 5 ml of liquid LB with the required antibiotics by shaking them at 37 °C for 16 hours before isolation. The instruction manual of the miniprep kit was applied after the 16 hours of inoculation.

2.2.12. Transfection of mammalian expression vector to N2a cells

Transfection is a process that artificially introducing the DNA or RNA into cells. Lipofectamine™ LTX with Plus™ reagents from Invitrogen® was used for introducing the fluorescently labeled Gα genes into N2a cells. 65000-90000 N2a cells were seeded on a 35 mm glass-bottom dish and they were grown in the growth medium

for 24 h before the transfection. In the following day, 100-300 ng genes found in mammalian expression vector were diluted in 100 µl of OptiMEM and mix thoroughly. 4 µl of Plus™ reagent was added to OptiMEM-DNA mixture. The mixture then was incubated at room temperature for 15 min. During the incubation, 4 µl Lipofectamine LTX was diluted in 100 µl of OptiMEM and added to the first mixture at the end of incubation. The final mixture was incubated at room temperature for 15 min. At that time, the medium of cells was removed and washed with 1 ml of sterile 1X PBS solution. Afterward, 800µl OptiMEM was added into the cells. At the end of incubation, the lipofectamine-DNA mixture was added on the cells. Cells were incubated at 37 °C for 3 hours. At the end of the process, the growth medium was added to incubated cells. Cells were grown at 37 °C for two days before imaging.

2.2.13. Imaging with Spinning Disc Confocal Microscope

Imaging of the cells was performed by Leica DMI4000 B equipped with Andor DSD2 spinning confocal microscope with 63X oil NA 1.4 objective lens. Andor DSD2 spinning disc confocal microscope has an excitation range of 370-700 nm and an emission range of 410-750 nm with a maximum of 22 frames per second. To reject the non-focused light, it has many rotating holes inside the disc. In addition, rotating holes provide focusing the light to the sample directly. This ensures sharper and detailed images (Figure 2.2).

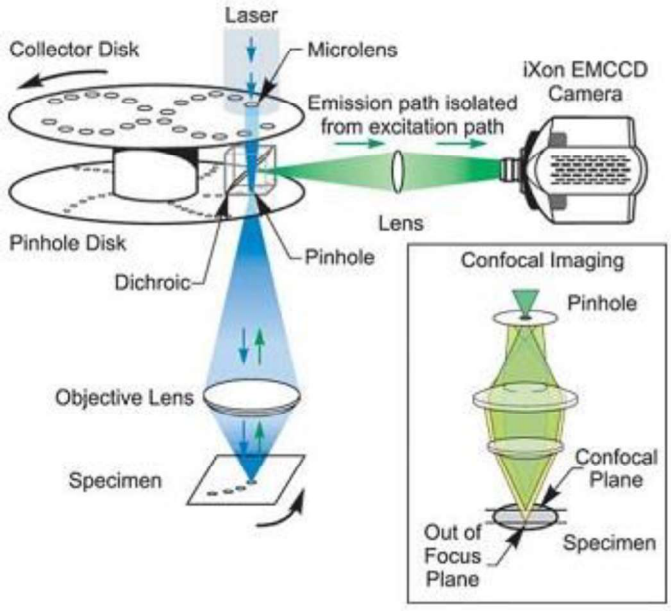


Figure 2.2: Schematically explained spinning disc confocal microscopy technology. (taken from Stehbens, Pemble, Murrow, & Wittmann, 2012)

The green signal from EGFP fluorophore was excited with the range of 464-500 nm wavelengths and emittion was collected in the range of 500-550 nm wavelengths. The red signal from mCherry fluorophore was excited at the range of 561-605 nm wavelengths and emittion was collected in the range of 600-650 nm wavelengths. In FRET analysis, cells were excited at the range of 464-500 nm wavelengths and emittion was collected in the range of 600-650 nm wavelengths.

2.2.14. Image Analysis with Pix-FRET Program

FRET method ensures the investigation of the interaction between proteins of interests. Although FRET has many advantages over the other protein-protein interaction methods, spectral bleed-through (SBE) is an important concern for FRET. Spectral overlap forms when acceptor and donor molecule excitation and emission wavelengths overlap and this overlapping could lead to false FRET signals. In this

study, the pixFRET program was used. PixFRET provides elimination of false FRET signals by analysis of three groups which are the only donor, only acceptor and both donor and acceptor, individually (Feige, Sage, Wahli, Desvergne, & Gelman, 2005).

In the first group, donor SBT was calculated. Donor-labeled genes were transfected to live cells and images were taken from FRET, and donor channels. In the donor channel, fluorophores were excited with the donor wavelengths and the signal from donor spectra was collected.

In the second group, acceptor SBT was calculated. Acceptor-labeled genes were transfected to cells and imaged with both FRET and acceptor channels. In the acceptor channel, fluorophore was excited with the acceptor wavelengths and signals were collected form acceptor spectra.

In the last group, both donor- and acceptor-labeled genes were transfected to live cells. Images were taken from three channels including FRET, donor, and acceptor. In the FRET channel, fluorophores were excited with the donor wavelengths and signals were taken from acceptor spectra. Using these three groups, the PixFRET algorithm eliminates bleed throughs and calculates the FRET efficiency for each pixel of the images (Feige *et al.*, 2005).

2.2.15. Functional Analysis with cAMP-Glo™ Assay

Functional analysis of fluorescence protein labeled Gα proteins was performed with the cAMP-Glo™ assay developed by Promega (WI, USA). Principle of this assay based on the measurement of cAMP concentration in cells. In the cells, if cAMP concentration rises, cAMP binds the protein kinase A (PKA). PKA has two regulatory and two catalytic components. If the cAMP absence in the cells, remaining PKA in an inactive form. The regulatory subunit undergoes a conformational change and releases its catalytic subunits. Free catalytic subunits catalyze the transfer of terminal

phosphate of ATP to the substrate of protein kinase A by consuming the ATP molecule. The level of unused ATP is defined by using luciferase-based Kinase-Glo reagent. The decrease in ATP concentration can be monitored as light. Luminescence is inversely proportional to cAMP levels in the cells. If the cAMP level decreases, the signal of the luminescence will increase (Figure 2.3).

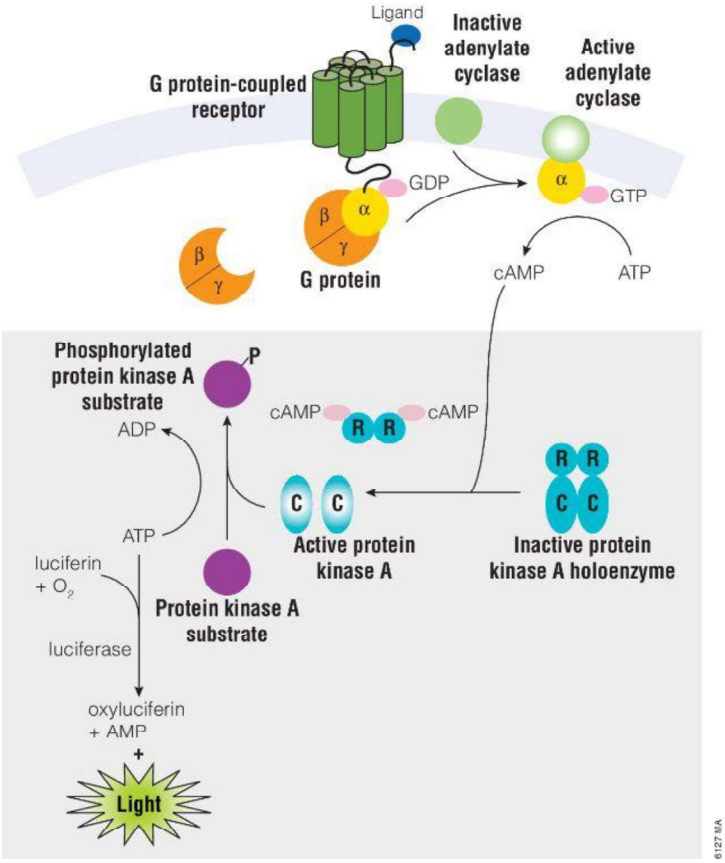


Figure 2.3: Illustration of cAMP production and the working principle of the cAMP-Glo assay (taken from www.promega.com).

In this study, the cAMP-Glo assay was performed to test the activity of fluorescently labeled inhibitory G protein (Gαi). As mentioned in section titled "G protein signaling

pathway through the Gαi and Gαs", Gαi protein is activated by Dopamine 2 receptor. Activation of Gαi inhibits the activity of adenylate cyclase enzyme which provides the production of cAMP in cells briefly, Gαi activation reduces the cAMP concentration in cells. According to this information, the cAMP-Glo assay will give high luminescence signal when applied to Gαi proteins.

To perform a cAMP-Glo assay to test the activity of Gαi proteins, approximately 120.000 N2a cells were seeded to 35 mm glass-bottom dish and incubated at 37 °C for 24 hours for growing the cells. After 24 h, the desired plasmid containing fluorescently labeled Gαi genes were transfected into N2a cells and incubated at 37 °C for 24 h. The next day, transfected cells were lifted and counted. Approximately 10.000 cells were seeded into 96 well plates. Cells again incubated at 37 °C for 24 h. In the following day, 20 µl of 20 µM forskolin (Sigma-Aldrich, #66575-29) diluted in 1X induction buffer (formulation in Appendix A) was added to all wells to increase the cAMP level to a certain level. Cells were incubated at 37 °C for 15 min. Then, 20 µl of 10 µM Quinpirole (Sigma-Aldrich, #73625-62) in induction buffer which is Dopamine 2 agonists used in literature was applied to all wells and incubated at 37 °C for 15 min (Eagle *et al.*, 2014). Then, assay protocol was applied according to the supplier's manual

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Visualization of mEGFP and mCherry fluorophore-labeled Gαi1 gene products

In this study, the homodimerization of Gαi1 protein was studied with FRET method. In the FRET method, dimerization is displayed as an energy transfer from donor to acceptor fluorophore protein. For the FRET, the intended protein genes should be labeled with donor and acceptor protein genes. mEGFP and mCherry fluorescent proteins were selected for the donor fluorescent protein and acceptor protein, respectively. These two fluorescent proteins demonstrate the favorable spectral overlap between donor emission and acceptor absorption. This is affirmed by the Förster radius R_0 = 5.1 nm. This value is highly compatible with the commonly used FRET pair, CFP-YFP. In addition, mEGFP has a large Stokes shift which is the difference between the band maxima of the absorption and emission spectra of the electronic transition. This large stokes shift provides very low crosstalk between donor and acceptor channels. The crosstalk index of mCherry-mEGFP FRET pair is very low among the other FRET pairs (Albertazzi, Arosio, Marchetti, Ricci, & Beltram, 2009). Moreover, this FRET pair has a low sensitivity to halide ions and proton and this provides the using of a number of different experimental conditions and highly variable intracellular environment of this pair (Arosio *et al.*, 2007). Finally, mEGFP-mCherry pair supplies a strong signal during *in vivo* FRET measurements (Bajar, Wang, Zhang, Lin, & Chu, 2016).

3.1.1. Tagging Gαi1 gene from A121-E122 and L91-K92 with mEGFP and mCherry fluorescence protein gene

For the construction of fluorescently labeled Gαi1, besides the G60-Y61 position that has been already tagged with both mEGFP and mCherry, two new positions were selected. One of them was in the loop, connecting helices A and B (L91-K92) and the other one was in the loop connecting the helices B and C (A121-E122) (see Figure 3.1). These domains had been previously studied for the insertion of sequences into Gαi1 without altering the biochemical and catalytic characteristics of the protein (Bünemann, Frank, & Lohse, 2003; Galés *et al.*, 2006). Agarose gel images of results of the PCR integration of EGFP and mCherry gene into Gαi1-wt can be seen in figure 3.2 and figure 3.3.

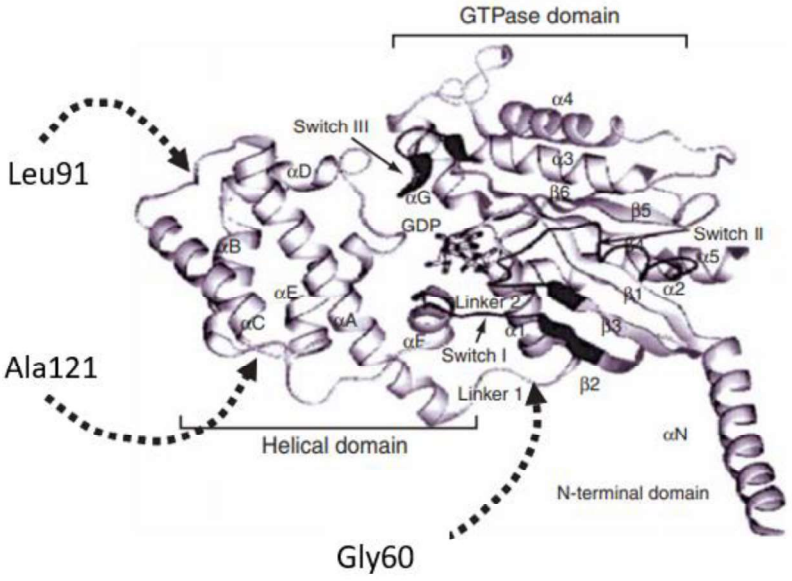


Figure 3.1:Three positions of the fluoresence protein tagging in the FRET study (Taken from **Galés et al., 2006)**.

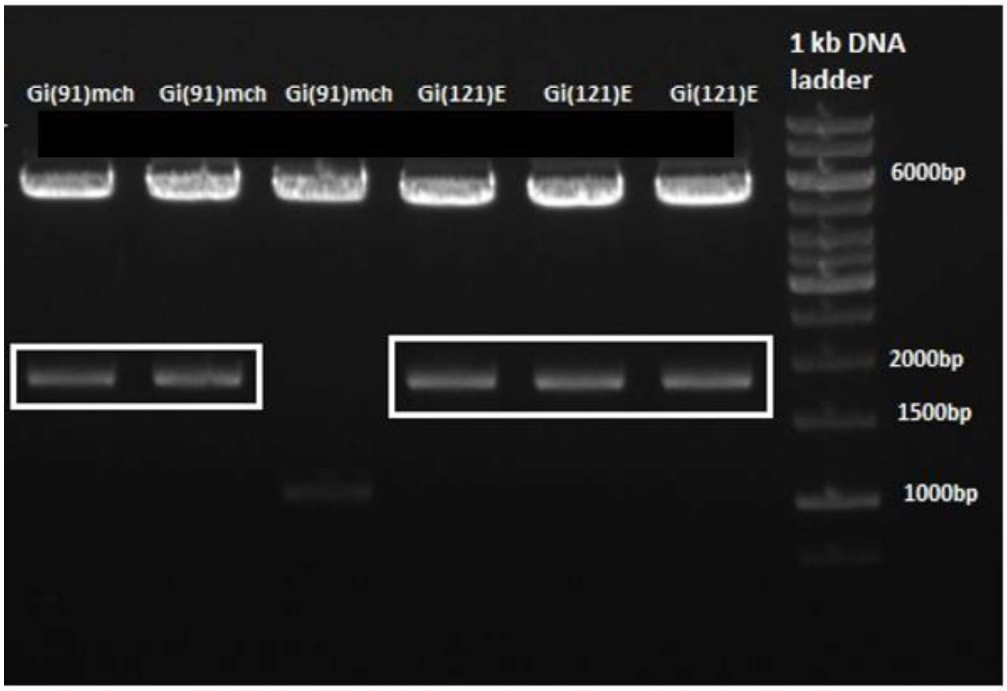


Figure 3.2: Agarose gel electrophoresis image of Gαi1 genes with mEGFP and mCherry in pcDNA 3.1 (-) vectors.Gαi1-wt gene is 1065 bp, Gαi1-mCherry is 1812 bp and Gαi1-mEGFP is 1821 bp.

Fluorescence protein genes inserted into Gαi1 gene was shown in white rectangles matching the expected band size.

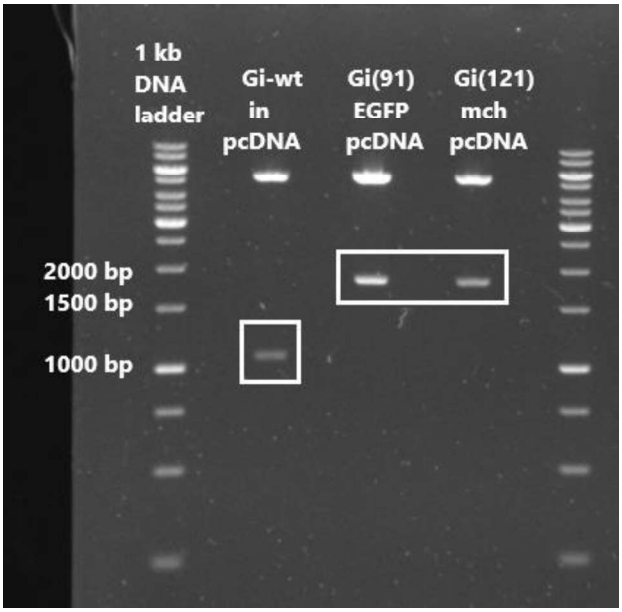


Figure 3.3: Agarose gel electrophoresis image of Gαi1 genes protein with mEGFP and mCherry.Gαi1-wt gene is 1065 bp, Gαi1-mEGFP is 1821 bp and Gαi1-mCherry is 1812 bp.

Fluorescence protein genes which inserted into Gαi1 gene were shown in white rectangles. The expected Gαi1-mCherry gene was 1812 bp and Gαi1-mEGFP gene was 1821 bp. The bands marked with white rectangles demonstrates the successful fluorescent protein labeled Gαi1 genes in pcDNA 3.1(-) vector. The upper bands represented the remaining pcDNA sequence after digestion, and as expected they were 5427 bp. The plasmids carrying these fusion proteins were sequenced for the final confirmation. According to sequencing results, no mutation was observed in these genes. The confirmed coding sequences of Gαi1 and fusion proteins were given in Appendix D. In addition to the position A121-E122 and L91-K92, mEGFP and mCherry genes were already inserted to position G60-Y61 in Gαi1 gene. These sequences were also verified and prepared for further experiments.

3.1.2. Tagging Gαi1 gene at G60-Y61,L91-K92 and A121-E122 with the N-EGFP & C-EGFP fragments

Bimolecular fluorescence complementation assay based on the complementation of two non-fluorescent protein pieces when the targeted proteins interact with each other (see Figure 1.6). In this study, non-fluorescent mEGFP protein pieces were used for the BiFC assay. Full-length mEGFP gene was split into two non-fluorescent pieces as N- and C-terminal fragments. These pieces correspond to the first 157 amino acid of mEGFP(1-157) called as N-EGFP and residues 158 to end of C-terminus (159-240) named as C-EGFP (Barnard & Timson, 2010).

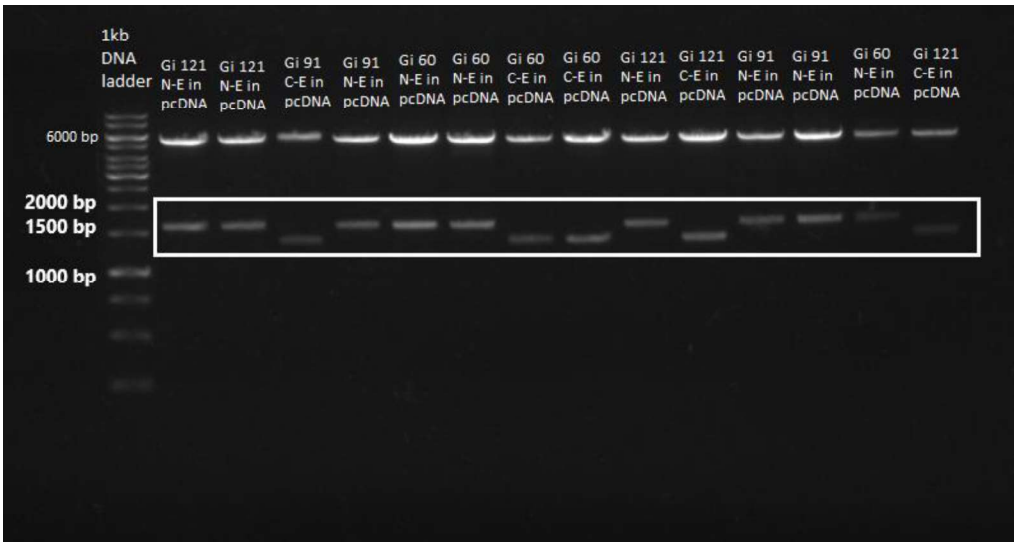


Figure 3.4: N-EGFP and C-EGFP labeled Gαi1 genes in pcDNA 3.1 (-) **.** N-EGFP labeled Gαi1 gene was 1575 bp and C-EGFP labeled Gαi1 was 1344 bp.

Correct band sizes correspounding to tagged Gαi1 gene were shown in white rectangle. Expected N-EGFP labeled Gαi1 gene size was 1575 bp and C-EGFP tagged Gαi1 protein was 1344 bp. The upper bands represented the remaining pcDNA sequence after digestion, and as expected were 5427 bp. According to gel result as seen in Figure 3.4, Gαi1 gene was succesfully labeled with split mEGFP protein parts

at all positions. After the confirmation of size in the gel, genes were sequenced. All sequences were verified (see in Appendix D).

3.1.3. Visualization of mEGFP labeled Gαi1 gene products at positions G60-Y61, L91-K92 and A121-E122

Gαi1 gene found in pcDNA 3.1(-) (mammalian expression vector) was labeled from two different positions with mEGFP fluorescence protein gene in this study. Full length mEGFP was inserted at three different positions as G60-Y61, L91-K92, and A121-E122 into Gαi1 gene. These mEGFP tagged plasmids were transfected to N2a cells which were derived from *Mus musculus* neuroblastoma cells, by transfection to confirm the expression and localization of proteins. N2a cells were imaged using Leica DMI 4000 equipped with Andor DSD2 spinning disk confocal microscope with 63X oil NA1.4 objective. Images were taken from G60-Y61, L91-K92 and A121-E122 labeled mEGFP fluorescent protein using only the mEGFP channel at the excitation of 484 nm (Figure 3.5).

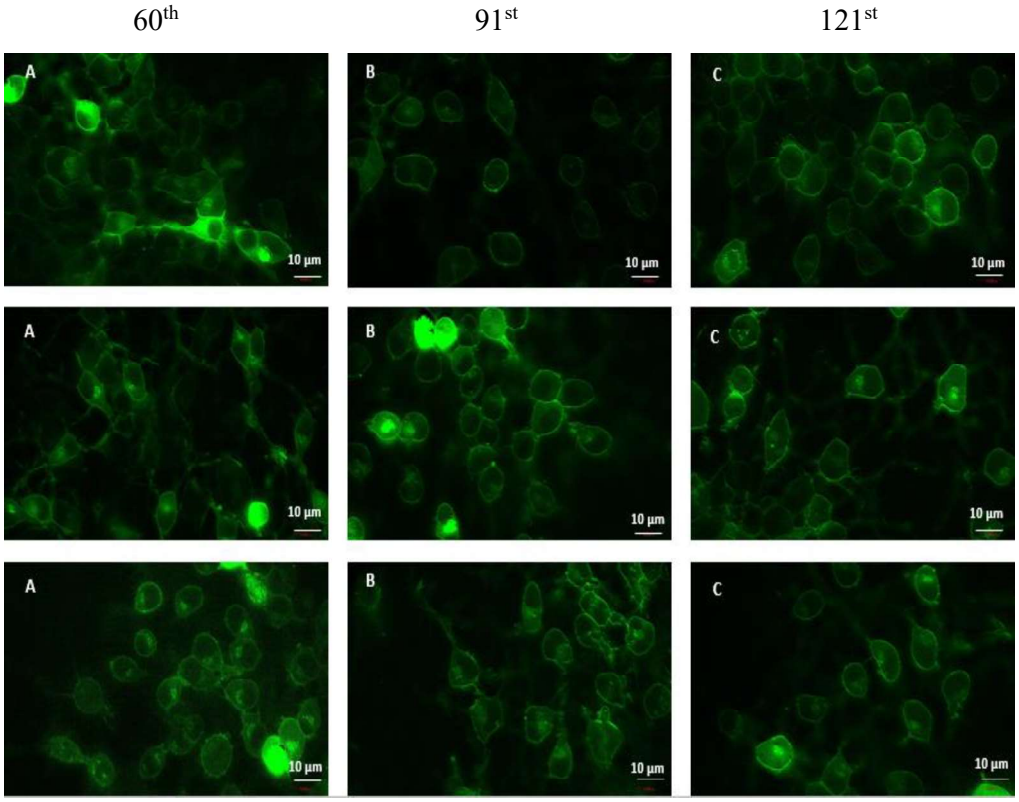


Figure 3.5: Confocal microscopy images of mEGFP labeled Gαi1 gene. A) position of G60-Y61 Gαi (60)-mEGFP, B) position of L91-K92, Gαi (91)-mEGFP and C) position of A121-E122, Gαi (121) -mEGFP. Images were taken from the mEGFP channel with 63X oil NA1.4 objective. Intensity 50%, excitation time 500 ms.

According to literature Gαi1 could be found in the centrosome, cytosol as well as the cell membrane where colocalizes with GPCR, RIC8A and RGS14 proteins (Cho & Kehrl, 2007). All three of the labeled Gαi1 gene products (60^{th}, 91^{st}, and 121^{st} amino acid positions) showed mEGFP signal on the cell membrane beside the other cellular compartments as expected. As the localization of these proteins were convenient as expected Gαi1 localization, they were used for further experiments.

3.1.4. Visualization of mCherry labeled Gαi1 gene at positions G60-Y61, L91-K92, and A121-E122

Gαi1 gene was labeled with mCherry at three different positions (G60-Y61, L91-K92 and A121-E122) and immaged using Andor DSD2 spinning disk confocal microscope with 63X oil NA1.4 objective (Figure 3.6). Images were taken using only mCherry channel which excited at 583 nm.

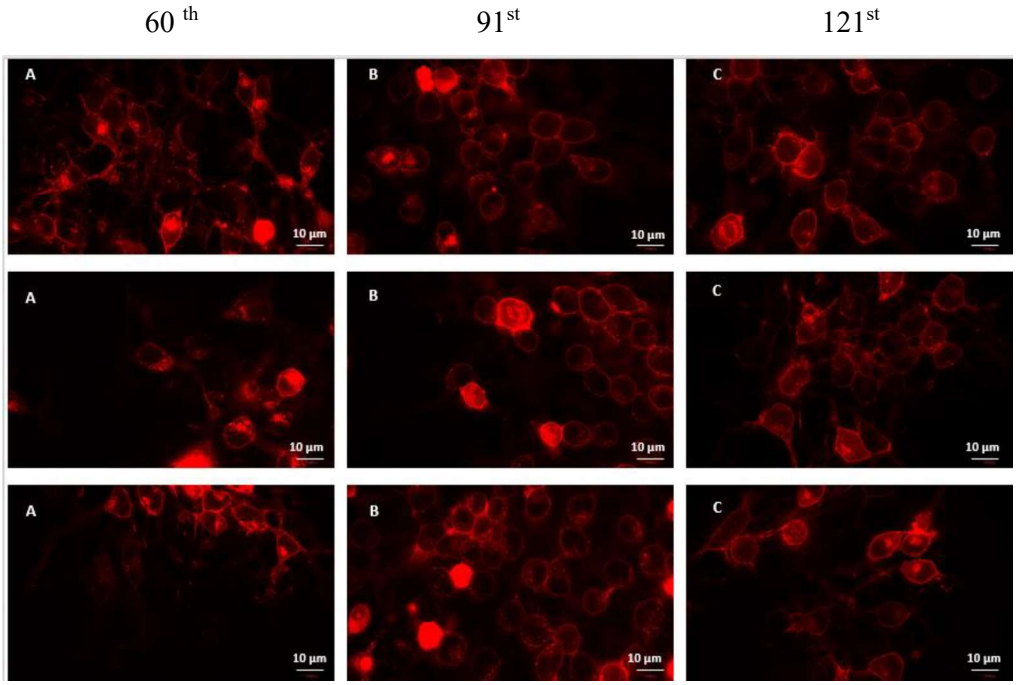


Figure 3.6: Confocal microscopy images of mCherry labeled Gαi1 genes**.** A) Position of G60-Y61 Gαi1(60)-mCherry, B) position of L91-K92, Gαi1(91)-mCherry and C) position of A121-E122, Gαi1(121)-mCherry. Images were taken from the mCherry channel with 63X oil NA1.4 objective. Intensity 100 %, excitation time 1000 ms.

Images showed that mCherry fluorescent protein which was inserted at various positions in Gαi1 gene properly expressed and localized on plasma membrane. Based on the Figure 3.6, these constructs were acceptable for FRET studies according to expression and localization in live cells.

3.2. Co-localization and FRET studies of labeled Gαi1 gene at different positions

For the FRET method, a targeted gene which is Gαi1 was labeled with both donor (mEGFP) and acceptor (mCherry) fluorescent protein genes at three different positions which were G60-Y61, L91-K92, and A121-E122. FRET depends on the distance between the targeted proteins. In order to determine an optimum position, mEGFP and mCherry tagged Gαi1 protein were co-transfected to N2a cells in six different combinations These six different combinations were:

- Gαi1(60)-mEGFP + Gαi1(60)-mCherry
- Gαi1(91)-mEGFP +Gαi1(91)-mCherry
- Gαi1(121)-mEGFP + Gαi1(121)-mCherry
- Gαi1(60)-mEGFP + Gαi1(91)-mCherry
- Gαi1(60)-mCherry +Gαi1(121)-mEGFP
- Gαi1(91)-mCherry +Gαi1 (121)-mEGFP

Images were taken using EGFP, mCherry and FRET channels separately. Figure 3.7 demonstrates the FRET studies at the combination of the same positions and figure 3.8 shows the combination of different positions.

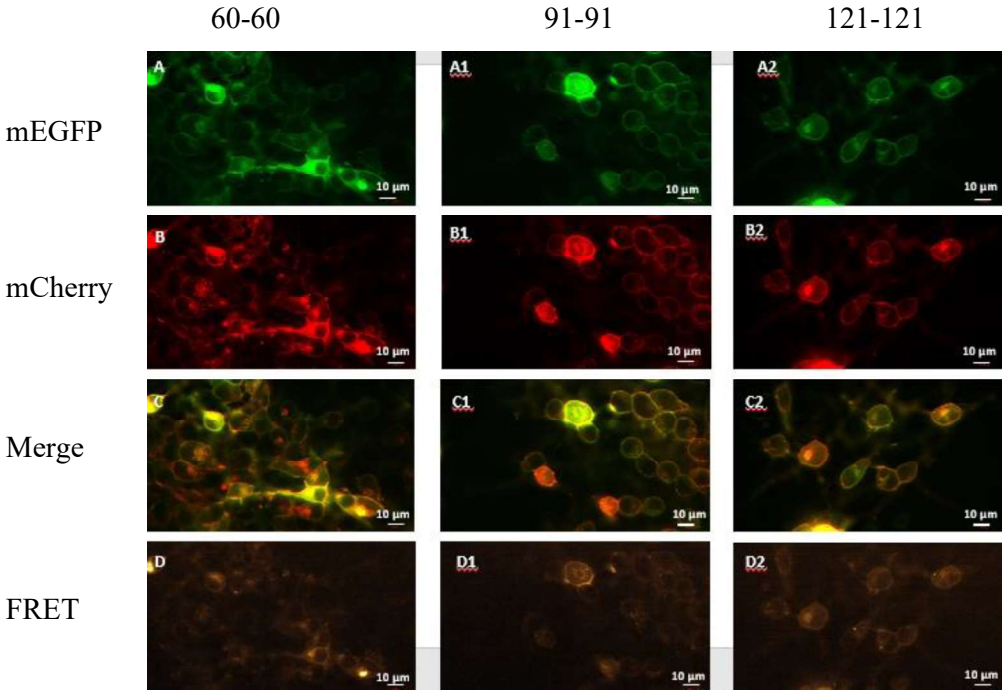


Figure 3.7: Fluorescent microscopy images of co-localization of Gαi1 gene products. A, B, C, and D) Gαi1 (60)-mEGFP + Gαi1 (60)-mCherry, A1, B1, C1, and D1) Gαi1 (91)-EGFP +Gαi1(91) -mCherry, A2, B2, C2, and D2) Gαi1(121)-mEGFP + Gαi1(121)-mCherry. A, A1 and A2 were taken using only mEGFP channel; B, B1 and B2 were taken using only mCherry channel; C, C1 and C2 are co-localization of mEGFP and mCherry channels; D, D1 and D2 were taken using FRET channel. Exposure time was 500 ms and intensity was 50 % for all channels.

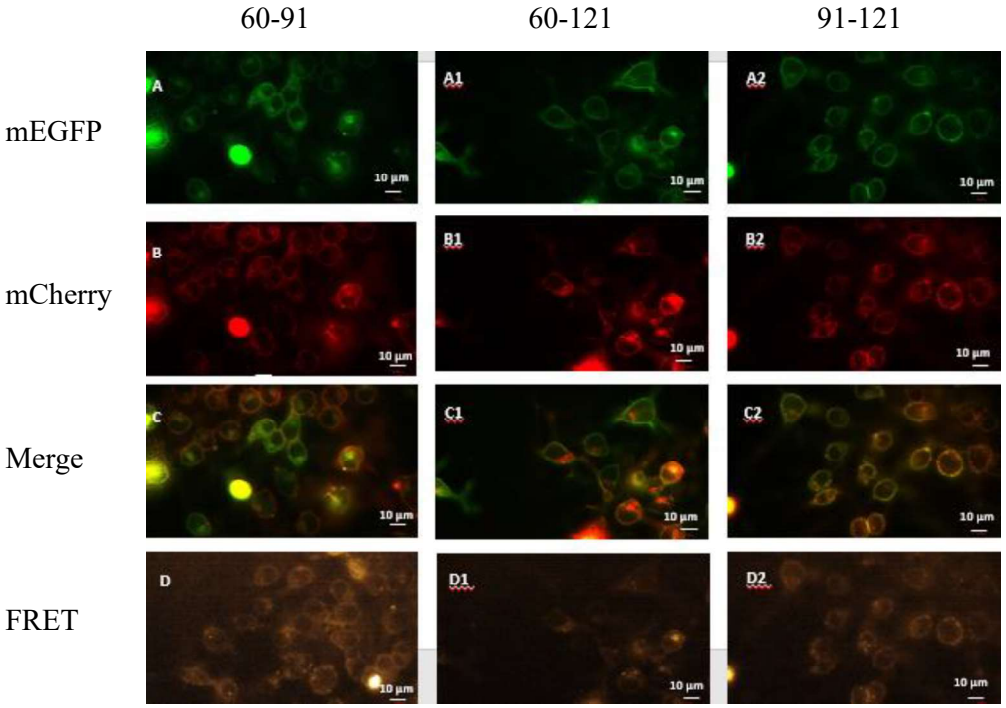


Figure 3.8: Fluorescent microscopy images of co-localization of Gαi1 gene products**.** A, B, C, and D) Gαi1(60)-mEGFP + Gαi1(91)-mCherry; A1, B1, C1, and D1) Gαi1(60)-mEGFP+Gαi1(121)-mCherry, A2, B2, C2, and D2) Gαi1(91)-mCherry + Gαi1(121)-mEGFP. A, A1 and A2 were taken using only mEGFP channel; B, B1 and B2 were taken using only mCherry channel; C, C1 and C2 were co-localization of mEGFP and mCherry channel, D, D1, and D2 was taken using FRET channel. Exposure time was 500 ms and intensity was 50 % for all channels.

According to figure 3.7 and figure 3.8, all fusion proteins expressed in the cell membrane as well as the cytosol and other cellular compartments when they were co-transfected into N2a cells. Images were taken using only mEGFP, only mCherry and only FRET channels with 50% intensity and 500 ms of exposure time. In FRET channel, flourescence intensities of 30 cells of these six combinations were analyzed to determine the position that supplied higher FRET

signal by Image J program (see Appendices H). According to graphic in the appendices H, the position of 121-121 had higher fluorescence intensity in FRET channel when compared the other combinations. Thus, it was decided to continue with the Gαi1(121)-mEGFP and Gαi1(121)-mCherry construct for further FRET studies.

3.3. Detection of Homodimerization of Gαi1 protein products labeled at position A121-E122 with FRET method

To investigate homodimerization of Gαi1 with FRET method, Gαi1 gene was labeled at position Ala121-Glu122 with both mCherry and mEGFP fluorescent protein genes. This position was selected as a result of localization and intensity analysis presented in figure 3.7 and figure 3.8. For FRET analysis, cells were imaged under three sets of conditions. The main aim of these three sets was to determine bleed-through by using the PixFRET program. Representative images of bleed-through signal and FRET was shown in Figure 3.9.

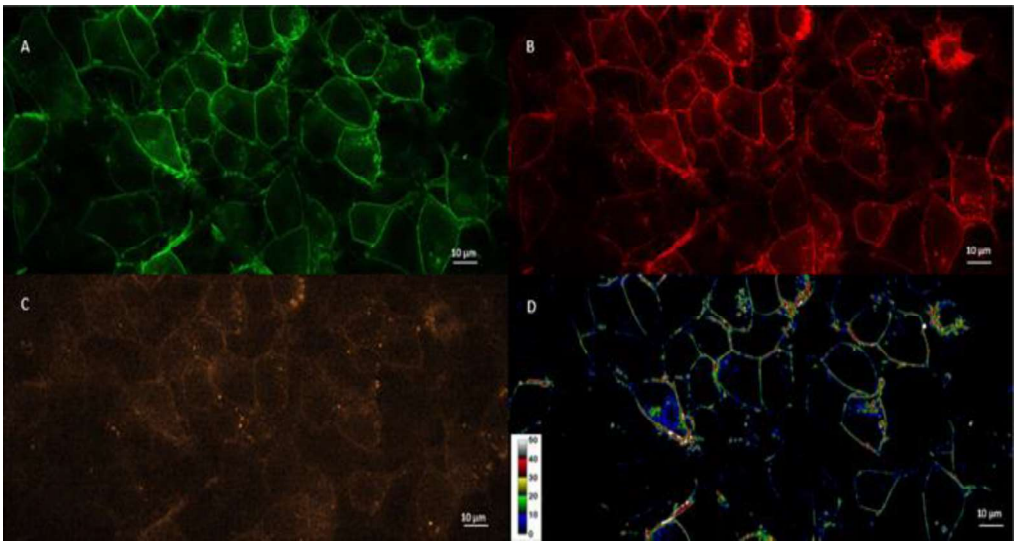


Figure 3.9: Confocal fluorescent microscopy image FRET study of Gαi1 protein. N2a cells co-transfected with 200 ng of Gαi1(121)-mEGFP and Gαi1(121)-mCherry A) Demontrates mEGFP channel excitation at 482 nm. Exposure time 0.7 s, intensity 100% B) Demonstrates mCherry channel excitation at 583 nm. Exposure time 0.7 s,

intensity 50% C) Demonstrates FRET channel. Exposure time 0.7 s, intensity 50% D) Demonstrates FRET signal efficiency. Color blue represents 0-10%, green 11-20%, yellow 21-30%, red 31-40%, and white 41-50% FRET efficiency.

Approximately 20 cells were imaged for the FRET analysis. These cells analyzed by PixFRET software to normalize bleed-throughs and determine the FRET efficiency ranges (1-10%, 11-20%, 21-30%, 31-40% and 41-50%). Images were colored to represent the efficiency of FRET and localization of tagged proteins in N2a cells. FRET efficiency was grouped into five different groups represented by various colors indicated in the calibration bars on the images. Color blue represents 1-10%, green 11-20%, yellow 21-30%, red 31-40%, and white 41-50% FRET efficiency. The first group representing 1-10% FRET efficiency generally courrespounds to long range background FRET signal. On the other hand, the percentage of 11-50% indicates the close proximity FRET signal that constitutes from the interaction of target proteins. In addition, the maximum actual FRET signal was represented in the 41-50% group and indicated as white color. When cells analyzed as presented in Figure 3.9, both Gαi1(121)-mEGFP and Gαi 1(121)-mCherry proteins were localized on the cell membrane of N2a cells as expected. FRET efficiency images Figure 3.9D also demonstrate that the strongest FRET signal was observed on the cell membrane (white color on the cell membrane). These images strongly suggests homodimerization of Gαi1 in live cells.

3.4. Effect of the wild-type Gαi1 protein on the homodimerization of tagged Gαi1 proteins

In section 3.1.5, homodimerization of Gαi1 protein which was labeled with mEGFP and mCherry fluorescence proteins at position A121-E122 was displayed with the FRET method. To further analyse this homodimerization, the effect of Gαi1-wt protein on the homodimerization was studied. In accordance with this purpose, two sets of FRET studies were performed. In the first set, only FRET pair which are

Gαi1(121)-mEGFP and Gαi1(121)-mCherry gene in mammalian expression vector pcDNA3.1(-) co-transfected into N2a cell to observe the interaction of these proteins. In the second sets, FRET pairs transfected with the Gαi1-wt gene. The main aim for addition of five times more wild-type protein gene into FRET study was to test the change in FRET efficiency of the homodimerization of Gαi1. A decrease in FRET efficiency would indicate interaction of wild-type protein with tagged Gαi1, thus decreasing the productive FRET pairs. Figure 3.10 had the representative images taken from FRET pair transfected and FRET pair + wild-type Gαi1 gene transfected cells.

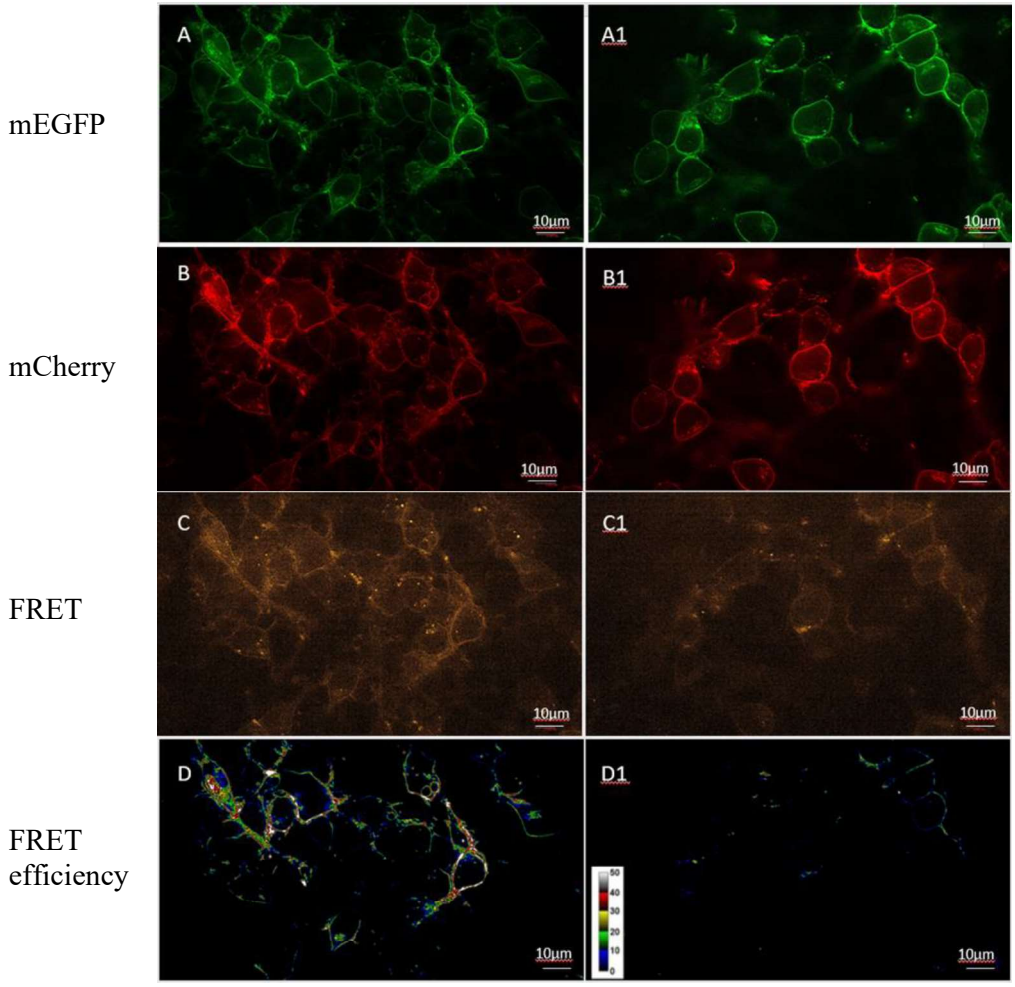


Figure 3.10: FRET stuedies of Gαi1 homodimerization and addition of Gαi-wt gene. N2a cells co-transfected with Gαi1 genes. A, B, C, and D had 200 ng of Gαi1(121)-mEGFP + Gαi1(121)-mCherry, A1, B1, C1 and D1 had 200 ng of Gαi1(121)-mEGFP

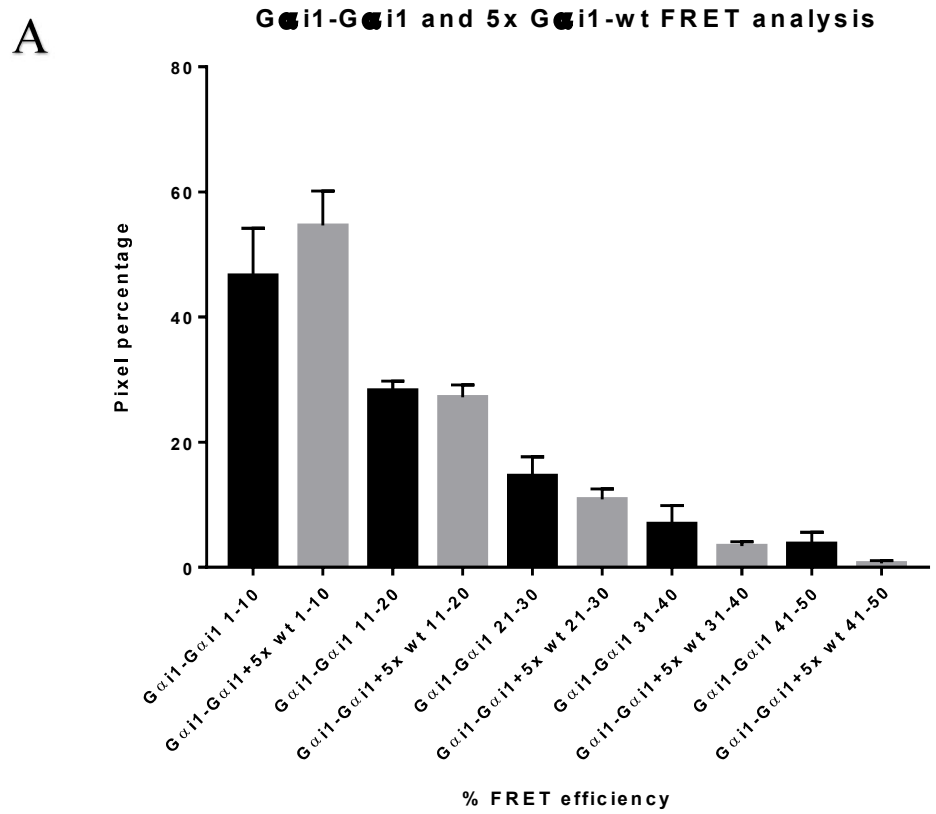
+ Gαi1(121)-mCherry plus1000 ng Gαi1-wt. A, A1) Demonstrates mEGFP channel excitation at 482 nm. Exposure time 0.7 s, intensity 100%; B, B1) Demonstrates mCherry channel excitation at 583 nm. Exposure time 0.7 s, intensity 50%; C, C1) FRET channel, exposure time 0.7 s, intensity 50%; D, D1) Demonstrates FRET Efficiency. Color blue represents 0-10%, green 11-20%, yellow 21-30%, red 31-40%, and white 41-50% FRET Efficiency.

Images of tagged Gαi1-Gαi1 and tagged Gαi1-Gαi1 + Gαi1-wt showed that both Gαi1(121)-mEGFP and Gαi1(121)-mCherry proteins were successfully expressed and co-localized on the cell membrane of N2a cells. In addition, wild-type Gαi1 proteins interacted with fluorescently labeled Gαi1 proteins and it was observed that, in the FRET efficiency representation (D parts) of Gαi1-Gαi1 + wild-type Gαi1 images, there was a significant decrease in FRET efficiency when compared to FRET efficiency images of Gαi1-Gαi1 interaction.

3.4.1. FRET analysis of homodimerization of Gαi1 protein and effect of wild-type Gαi1 protein by PixFRET plugin

FRET analysis was performed by pixFRET plugin of ImageJ program to allow normalized FRET by calculating the images pixel by pixel the images of sample acquired in a three channels setting (see section 2.2.14). For quantification of FRET, a region of interest on the cell was drawn to obtain histogram giving the pixel counts. Then, FRET efficiencies at these pixels were recorded by using these histograms. Quantification analysis of FRET for homodimerization of Gαi1 protein and the effect of wild-type Gαi1 protein on this homodimerization was performed by pixFRET analysis. Specific range of FRET efficiencies and percentage of every range (1-10%, 11-20%, 21-30%, 31-40% and 41-50%) were computed.

A


Gαi1-Gαi1 and 5x Gαi1-wt FRET analysis
Pixel percentage
80
60
40
20
0
Gαi1-Gαi1 1-10
Gαi1-Gαi1+5x wt 1-10
Gαi1-Gαi1 11-20
Gαi1-Gαi1+5x wt 11-20
Gαi1-Gαi1 21-30
Gαi1-Gαi1+5x wt 21-30
Gαi1-Gαi1 31-40
Gαi1-Gαi1+5x wt 31-40
Gαi1-Gαi1 41-50
Gαi1-Gαi1+5x wt 41-50
% FRET efficiency

B

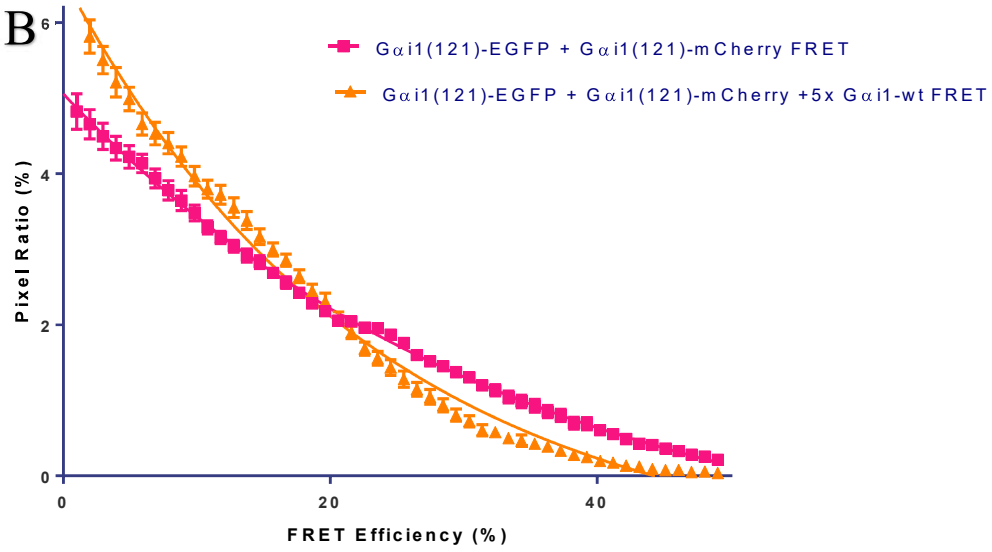

Gαi1(121)-EGFP + Gαi1(121)-mCherry FRET
Gαi1(121)-EGFP + Gαi1(121)-mCherry +5x Gαi1-wt FRET
Pixel Ratio (%)
6
4
2
0
0
20
40
FRET Efficiency (%)

Figure 3.11: FRET efficiency analysis. A) Analysis of approximately 30 cells by pixFRET plugin of Image J**.** For every efficiency ranges (1-10%, 11-20%, 21-30%, 31-40%, and 41-50%) histograms were obtainedmore separately; so that pixel counts of every range could be recorded. By comparing with the total pixel count giving FRET, the percentage of every range in the cells was detected. B) Demonstration of all data set with line graph.

FRET efficiency graphic was created with Graphpad prism 7.00 and data were analyzed by using an unpaired t-test (Figure 3.11 A). In the figure 3.11 B, cells were analyzed with the pixFRET plugin without seperation of range of FRET efficiencies (1-10%, 11-20%, 21-30%, 31-40%, and 41-50%). This graph demonstrated that FRET efficiency of cells according to pixel ratio of 1-50% percentage of efficiency using line graph. In this study, N2a cells were analyzed under two conditions +/- 5X Gαi1-wild type (Appendix D). Statistical analysis of figure 3.11A was done using unpaired t-test because this test is useful for comparing mean values of two unmatched groups.

According to the graphics presented in figure 3.11, it was concluded that adding five times more Gαi1-wt significantly decreased the FRET efficiencies of 11-50 % when compared to the homodimerization of Gαi1-Gαi1. In the range of 1-10 % in figure 3.11 A, FRET efficiency is higher in addition to Gαi1 wild-type. As mentioned in section 3.1.5, the range of 1-10% gives mostly background FRET signal, but the range of 11-50 % represents close proximity FRET signal. Higher pixel percentage in 1-10% FRET Efficiency when 5X Gαi1 wild-type added matches with our hypothesis suggesting the interaction of wild-type protein with tagged proteins thus decreasing actual FRET pairs. In addition to microscopy images, quantitatively FRET analysis also strongly supported the Gαi1 homodimerization in N2a cells.

3.5 FRET analysis of homodimerization of Gαi1 protein by microplate reader

FRET analysis of Gαi1 protein homodimerization was also studied with microplate reader quantitatively. In this study, Gαi1(121)-mEGFP, Gαi1(121)-mCherry and Gαi1(121)-mEGFP + Gαi1(121)-mCherry genes were transfected into approximately 120.000 N2a cells separately. In addition to FRET of Gαi1 genes, soluble mEGFP and mCherry which are found in pcDNA3.1(-) and membrane-targeted mCherry gene and Gαi1(121)-mEGFP genes were also transfected to N2a cells for negative control of FRET Efficiency. Besides this, the effect of 5X wild-type Gαi1 gene into homodimerization was also tested with microplate reader. FRET was analyzed with a microplate reader. mEGFP was excited at 488 nm and emission was recorded at 512 nm, mCherry was excited at 588 nm and emission was recorded at 615 nm. Finally, FRET pairs were excited at 488 nm and emission was recorded at 615 nm. These wavelengths were optimized in accordance to the fluorescence spectrum of each set (mEGFP, mCherry, and FRET) in microplate reader. Results were evaluated (Figure 3.12).

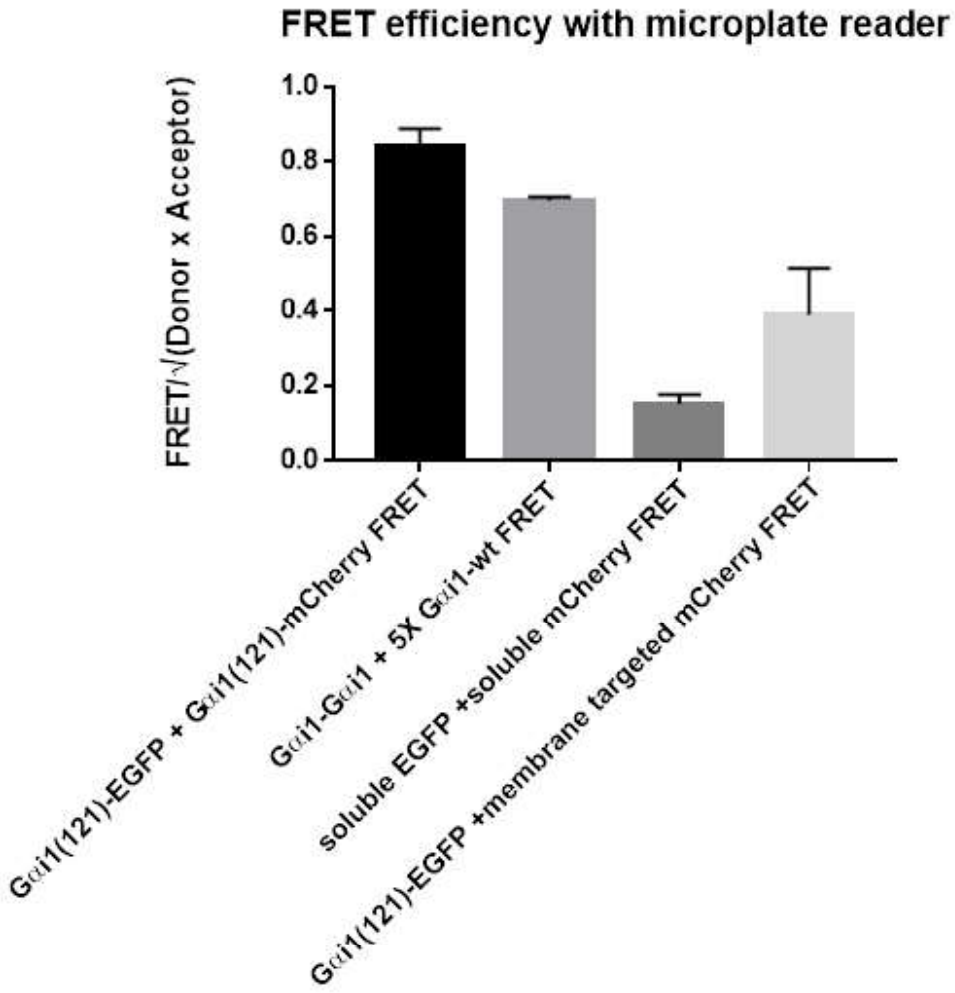


Figure 3.12: FRET analysis graphic of homodimerization of Gαi1(121)-mEGFP + Gαi1(121)-mCherry, Gαi(121)-mEGFP + Gαi(121)-mCherry + Gαi-wt, soluble mEGFP + soluble mCherry and Gαi(121)-mEGFP+membrane targeted mCherry genes with the multi-plate reader. Excitation time 1000 ms.

FRET was calculated using the following formula:

$$\frac{FRET}{\sqrt{(Donor * Acceptor)}}$$

Data were analyzed and the graph was constituted with an unpaired t-test in Graphpad Prism 7.00. According to graph 3.12, FRET efficiency optained due to homodimerization of Gαi1 protein was higher than the negative controls which were soluble EGFP - mCherry and membrane-targeted mCherry + Gαi1(121)-mEGFP FRET. In addition, it was found that the FRET efficiency of 5X Gαi1 wild-type was decreased compared to the Gαi1-Gαi1 FRET however p value was 0.059 (see appendix F). On the other hand, the FRET efficiency of both Gαi1-Gαi1 and Gαi1-Gαi1+Gαi1-wt were higher than the negative controls which were soluble mEGFP + soluble mCherry FRET and Gαi1(121)-mEGFP + membrane targeted mCherry FRET. These results support our findings, and we can conclude homodimerization of Gαi1 protein can be studied using microplate reader as well as pixFRET analysis.

3.6 Detection of Gαi1 protein homodimerization by Bimolecular Fluorescence Complementation Assay (BiFC)

In this study Gαi1 protein homodimerization was also investigated using Bimolecular Fluorescence Complementation Assay. Unlike FRET method this technique is a qualitative measure of protein-protein interaction. N-terminal mEGFP (1-158 aa) and C-terminal mEGFP (159-240 aa) tagged Gαi1 protein pairs at the position G60-Y61, L91-K92, and A121-E122 were co-transfected into the N2a cell. Images were taken using spinning disc confocal microscopy after 48-hour of transfection (Figure 3.13).

For the BiFC studies, various combinations of tagging position with N-EGFP and C-EGFP was tested with confocal fluorescence microscope. These combinations were:

- Gαi1(60)-N-EGFP + Gαi1(60)-C-EGFP
- Gαi1(91)-N-EGFP+Gαi1(91)-C-EGFP
- Gαi1(60)-N-EGFP+Gαi1(91)-C-EGFP
- Gαi1(60)-N-EGFP + Gαi1(121)-C-EGFP

- Gαi1(91)-N-EGFP +Gαi1(121)-C-EGFP
- Gαi1(121)-N-EGFP + Gαi1(121)-C-EGFP

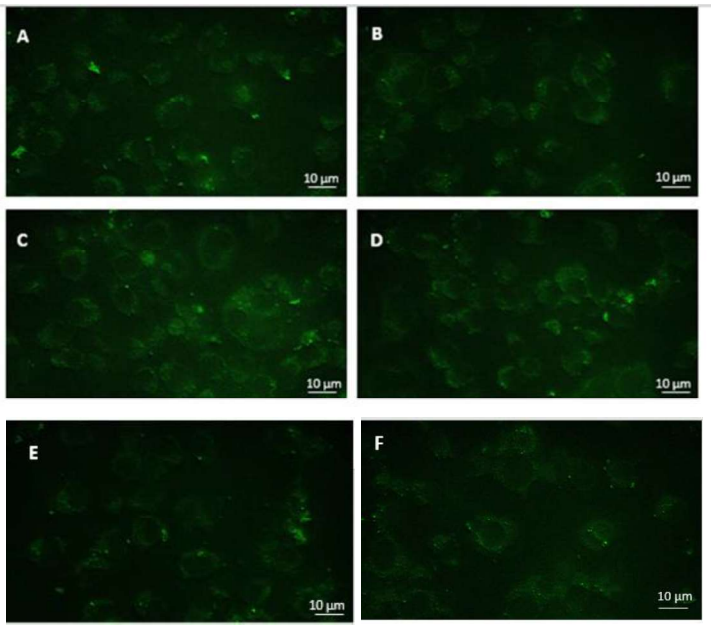


Figure 3.13: Fluorescence confocal microscope images of BiFC assay. N2a cells co-transfected with 500 ng of N-EGFP and C-EGFP labeled Gαi1 A) Gαi1(60)-Gαi1(60), B) Gαi1(60)-Gαi1(91) C) Gαi1(60)-Gαi1(121), D) Gαi1(91)-Gαi1(121), E) Gαi1(121)-Gαi1(121) and E) Gαi1(91)-Gαi1(91). All images were taken using EGFP chanel exposure time 1000 ms, intensity 100% 63x NA 1.4.

According to images, mEGFP signal was observed in all combinations. However, the signal was very low and localized intracellularly. Unexpectedly, very little or no EGFP signal was observed on the cell membrane. Following this unexpected observation, effect of agonist and antagonist of Dopamine 2 receptor (D2R) which was the receptor interacting with the Gαi1 protein in our study, were tested. To observe the effect of agonist and antagonist in BiFC, only one of the various combinations tested was chosen (Gαi1(121)-N-EGFP + Gαi1(121)-C-EGFP) considering the FRET studies with these same tagging positions. For this study, Quinpirole (Sigma, # Q111) as an

agonist and (R, S)-Sulpiride (Abcam, # 15676-16-1) as an antagonist was applied to cells. Representative images were presented in Figure 3.14.

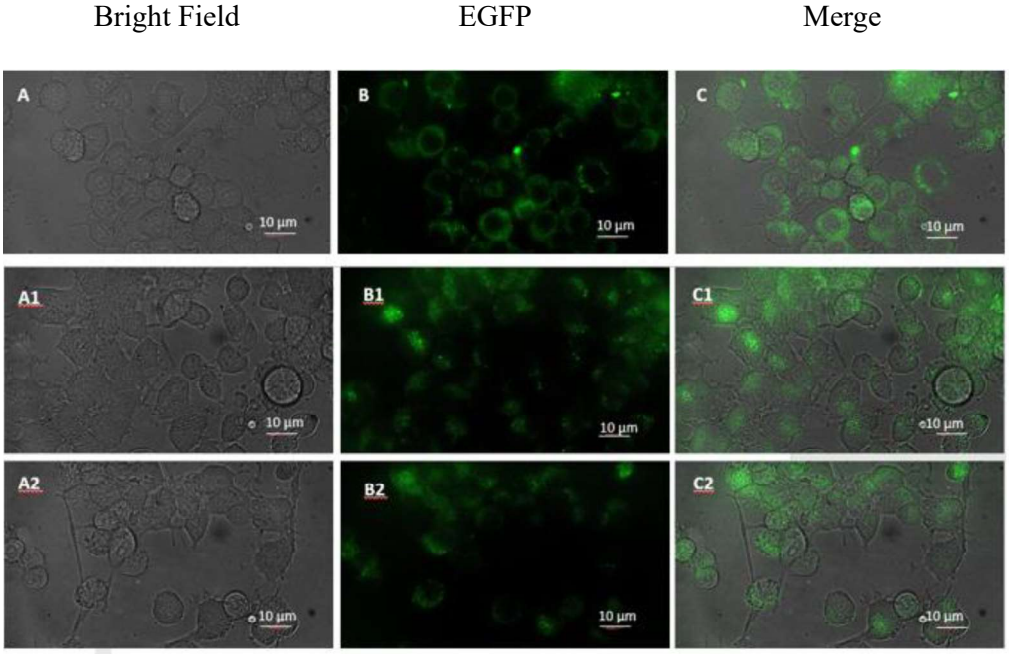


Figure 3.14: Fluorescence confocal microscopy images of BiFC method with the effect of agonist and antagonist. A, A1 and A2 bright field, B, B1 and B2 mEGFP chanel and C, C1 and C2 were merged of bright field and mEGFP channel. A, B and C) cells without any treatment of agonist and antagonist, A1, B1 and C1) Images taken from 10 µM of quinpirole treatment for 30 min., and A2, B2, and C2) Images taken from 100 µM (R, S)-Sulpiride treatment for 30 min. Excitation time 1000 ms, intensity 100%, 63x, NA. 1.4.

According to Figure 3.14, there was no significant alteration after the treatment of agonist and antagonist compared to untreated cells in terms of mEGFP signal and localization of this signal. This result could be due to insufficient time or amount of agonist and antagonist application to observe the effect of treatment. Another reason might be, the improper position of labeling for split mEGFP studies. It was interpretted that the positions suitable for FRET tagging might not be suitable for BiFC studies.

3.7 Functional analysis of fluorescently labeled Gαi1 using cAMP-Glo™ Assay

In order to demonstrate the functionality of mEGFP, mCherry and split mEGFP (N-EGFP and C-EGFP) labeled Gαi1 proteins cAMP-Glo assay was used. As mentioned in section 1.1.4, Gαi1 proteins were classified as inhibitory G-protein because of their effect on prevention of cAMP production in cells. The main principle of cAMP assay relies on the detection of the luminescence which is inversely proportional to cAMP level in cells. According to this assay, it was expected to observe a reduction of luminescence of fluorescently labeled Gαi1 protein in the manner of Gαi1-wt. For this purpose, the cAMP assay protocol was performed both wild-type Gαi1 genes and fluorescently labeled Gαi1 genes to observe similarity of the reduction of luminescence amount between wild type and labeled genes.

According to results, the reduction amount of luminescence signal between only forskolin applied cells and quinpirole applied cells was not significantly different unlike what was expected for wild-type Gαi1 (see appendix F). This condition was also observed in fluorescently labeled Gαi1. These unexpected results suggested that either the forskolin or quinpirole did not affect the cells. Thus, the functionality of fluorescently labeled Gαi1 genes could not be tested with the cAMP-Glo assay. The necessary ligands and a new kit was ordered and this assay will be repeated when the components are available. Nevertheless the positions used in this study were selected according to literature and were reported to be functional in other studies (Bünemann, Frank, & Lohse, 2003; Galés *et al.*, 2006)

CHAPTER 4

CONCLUSION AND FUTURE STUDIES

Gαi1 protein which is an inhibitory guanine binding protein was successfully tagged with both mEGFP and mCherry fluorescence protein gene at the position of L91-K92 and A121-E122 for the FRET studies. Successful tagging was confirmed by sequencing.

In addition, the Gαi1 protein gene was successfully labeled with both split EGFP fragments (N-EGFP and C-EGFP) at position G60-Y61, L91-K92 and A121-E122, these sequences were also verified by sequencing.

Confocal microscope images showed that mEGFP and mCherry tagged Gαi1 protein at position L91-K92 and A121-E122 were expressed and localized expectedly at the cell membrane.

The position A121-E122 was selected for the FRET studies because of the higher FRET signal when compared to other positions.

FRET efficiency images of Gαi1(121)-mEGFP and Gαi1(121)-mCherry demonstrate that strongest FRET signal was observed at the cell membrane. These images strongly suggests homodimerization of Gαi1 protein in live cells.

FRET Efficiency images of $G\alpha i_1$-$G\alpha i_1$ + Gαi wild type, showed that there was a significant decrease in FRET efficiency when compared to FRET efficiency images of $G\alpha i_1$-$G\alpha i_1$ interaction. In addition, FRET Efficiency calculation by PixFRET shows that there was significant decrease in percentage of 21-30%, 31- 40% and 41-50% pixels in $G\alpha i_1$-$G\alpha i_1$ + Gi wild type when compared to $G\alpha i_1$-$G\alpha i_1$ interaction. These results strongly suggest the $G\alpha i_1$-$G\alpha i_1$ protein interaction in N2a cells.

In Bimolecular fluorescence complementation assay (BiFC) N-EGFP and C-EGFP labeled Gαi1 proteins were co-transfected into N2a cells. Although, the signal of the EGFP and mCherry fused Gαi1 protein which labeled at the same position with the split EGFP gene observed on the cell membrane, signal of the split EGFP was not located at the membrane. In addition, there was no effect of the addition of agonist and antagonist on the signal of the split EGFP under the applied conditions.

In future,the functionality of labeled Gαi1 protein will be repeated with the cAMP-Glo assay.In addition, the expression levels of labeled Gαi1 protein will be compared to Gαi1-wt protein expression will be investigated.

APPENDICES

A. COMPOSITIONS OF SOLUTIONS

Table A. 1 Composition of D-MEM with high glucose

COMPONENT	CONCENTRATION (mg/L)
Amino Acids	
Glycine	30
L-Arginine hydrochloride	84
L-Cysteine 2HCl	63
L-Glutamine	580
L-Histidine hydrochloride-H_2O	42
L-Isoleucine	105
L-Leucine	105
L-Lysine hydrochloride	146
L-Methionine	30
L-Phenylalanine	66
L-Serine	42
L-Threonine	95
L-Tryptophan	16
L-Tyrosine	72
L-Valine	94
Vitamins	
Choline chloride	4
D-Calcium pantothenate	4

Folic acid	4
Niacinamide	4
Pyridoxine hydrochloride	4
Riboflavin	0.4
Thiamine hydrochloride	4
i-Inositol	7.2
Inorganic Salts	
Calcium chloride	264
Ferric nitrate	0.1
Magnesium sulfate	200
Potassium chloride	400
Sodium bicarbonate	3700
Sodium chloride	6400
Sodium phosphate monobasic	141
Other components	
D-Glucose (Dextrose)	4500
Phenol Red	15
Sodium pyruvate	110

Luria Bertani (LB) Medium

10 g/L Tryptone

5 g/L NaCl

5 g/L Yeast Extract

All of the components are dissolved in distilled H_2O. 20 g/L agar is added for solid medium preparation. The pH of the medium is adjusted to 7.0.

PBS buffer, 10X

11.5g Na2

80g NaCl 2g KCl

Dissolve in 1 liter of sterile, deionized water. The pH of 1X PBS will be 7.4.

1X TAE (Tris Base, Acetic acid, EDTA) Buffer

40mM Tris

20mM Acetic Acid

1mM EDTA

Dissolved in dH2O.

Table A.2 Composition of TFBI and TFBII

TFB I	Solution Concentration	Prepared Stock	For 100 mL Solution take from prepared stock
KOAc	30 mM	300 mM	10 mL
RbCl	100 mM	1000 mM	10 mL
$CaCl_2$	10 mM	1000 mM	1 mL
$MnCl_2$	50 mM	1000 mM	5 mL
Glycerol	15%	87%	17.2 mL

Complete the solution to 100 mL with distilled water and adjust pH to 5.8. After adjusting the pH, autoclave the solution or filter the solution using 0.45 mm filter.

TFB I	Solution Concentration	Prepared Stock	For 100 mL Solution take from prepared stock
KOAc	30 mM	300 mM	10 mL
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$CaCl_2$	10 mM	1000 mM	1 mL
$MnCl_2$	50 mM	1000 mM	5 mL
Glycerol	15%	87%	17.2 mL

Complete the solution to 100 mL with distilled water and adjust pH to 5.8. After adjusting the pH, autoclave the solution or filter the solution using 0.45 mm filter.

1X Induction Buffer

Krebs Ringer buffer, serum-free medium or 1X PBS containing 100µM Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl) imidazolidone] and 500µM IBMX (3-isobutyl1-methylxanthine).

B. MAPS OF THE MAMMALIAN EXPRESSION VECTOR

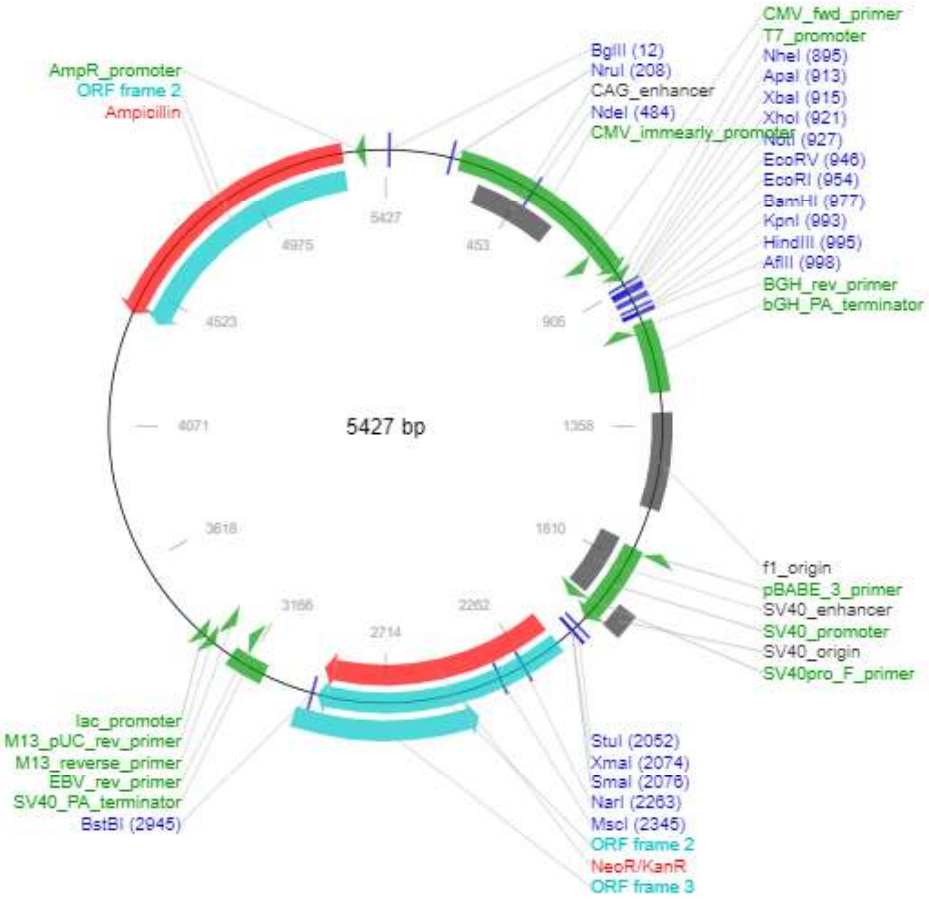

Figure B. 1 Map of mammalian expresion vector (pcDNA 3.1 (-)) with the CMV promoter (www.addgene.org)

C. PRIMERS

Table C.1: Primers that design for the mEGFP and mCherry labelling at the positions of A121-E122 and L91-K92 in the Gαi1 genes

Protein	Position	Primer	Sequence
Gαi1	L91-K92	EGFP/mCherry Forward primer	5'-TAG GGC TAT GGG GAG GTT GTC TGG AGG AGG AGG ATC TAT GGT GAGCAA GGG CGA GG-3'
		EGFP/mCherry Reverse primer	5'-AGT CAC CAA AGT CTA TCT TAG ATC CTC CTC CTC CAG ACT TGT ACA GCT CGT CCA TG-3'
Gαi1	A121-E122	EGFP/mCherry Forward primer	5'-AGA AGG CTT TAT GAC TGC ATC TGG AGG AGG AGG ATC TAT GGT GAG CAA GGG CGA GG-3'
		EGFP/mCherry Reverse primer	5'-TTA TAA CTC CAG CAA GTT CAG ATC CTC CTC CTC CAG ACT TGT ACA GCT CGT CCA TG-3'

Table C.2 : Primers designed for insertion of N-EGFP and C-EGFP into the Gαi1 genes.

Protein	Position	Primer	Sequence
Gαi1	G60-Y61	N-EGFP forward	5'-AAA TTA TCC ATG AAG CTG GTT CTGGAG GAG GAG GAT CTA TGG TGA GCA AGG GCG AGG-3'
		N-EGFP reverse	5'-CAC TCC TCT TCT GAA TAA GAT CCT CCT CCT CCA GAC TGC TTG TCG GCC ATG ATA TAG-3'
		C-EGFP forward	5'-GAA AAT TAT CCA TGA AGC TGG TTC TGG AGG AGG AGG ATC TAA GAA CGG CAT CAA GGT G-3'
		C-EGFP reverse	5'-TAC ACT CCT CTT CTG AAT AAG ATC CTC CTC CTC CAG ACT TGT ACA GCT CGT CCA TGC-3'
Gαi1	L91-K92	N-EGFP forward	5'-TAG GGC TAT GGG GAG GTT GTC TGG AGG AGG AGG ATC TAT GGT GAG CAA GGG CGA GG-3'
		N-EGFP reverse	5'-GTC ACC AAA GTC TAT CTT AGA TCC TCC TCC TCC AGA CTG CTT GTC GGC CAT GAT ATA G-3'
		C-EGFP forward	5'-CAT TAG GGC TAT GGG GAG GTT GTC TGG AGG AGG AGG ATC TAA GAA CGG CAT CAA GGT G-3'
		C-EGFP reverse	5'-AGT CAC CAA AGT CTA TCT TAG ATC CTC CTC CTC CAG ACT TGT ACA GCT CGT CCA TG-3'
Gαi1	A121-E122	N-EGFP forward	5'-AGA AGG CTT TAT GAC TGC ATC TGG AGG AGG AGG ATC TAT GGT GAG CAA GGG CGA GG-3'
		N-EGFP reverse	5'-CTC TTT ATA ACT CCA GCA AGT TCA GAT CCT CCT CCT CCA GAC TGC TTG TCG GCC ATG-3'
		C-EGFP forward	5'-GAA GAA GGC TTT ATG ACT GCA TCT GGA GGA GGA GGA TCT AAG AAC GGC ATC AAG GTG-3'
		C-EGFP reverse	5'-TTA TAA CTC CAG CAA GTT CAG ATC CTC CTC CTC CAG ACT TGT ACA GCT CGT CCA TG-3'

D. CODING SEQUENCES OF FUSION PROTEINS

Coding sequence of GNAI1 (Accession Number: DQ892385)

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTATTCAGAAGAGGAGTGTAAACAATACAAAGCA
GTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGG
AGGTTGAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGATGCACGCCAACTC
TTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCAGAACTTGCTGGA
GTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGTTTCAACAGATCC
CGAGAGTACCAGCTTAATGATTCTGCAGCATACTATTTGAATGACTTGGACAGA
ATAGCTCAACCAAATTACATCCCGACTCAACAAGATGTTCTCAGAACTAGAGTG
AAAACTACAGGAATTGTTGAAACCCATTTTACTTTCAAAGATCTTCATTTTAAAA
TGTTTGATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGGATTCATTGCTTCG
AAGGAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGACTACGACCTGGTTC
TAGCTGAAGATGAAGAAATGAACCGAATGCATGAAAGCATGAAATTGTTTGAC
AGCATATGTAACAACAAGTGGTTTACAGATACATCCATTATACTTTTTCTAAACA
AGAAGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCTCACTATATGCTATC
AAGAATATGCAGGATCAAACACATATGAAGAGGCAGCTGCATATATTCAATGTC
AGTTTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAATATACACCCACTTC
ACATGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGATGCTGTAACAGAT
GTCATCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTAG

Coding Sequence of Gαi1(60)-mCherry: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and red sequence represents mCherry sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTCTGGAGGAGGAGGATCTATGGTGAGCAAGGGC
GAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATG
GAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCG
CCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCC
TGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTA
CGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGG
CTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGA
CCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCG
GCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGG
AGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATC
AAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGAC
CACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACAT
CAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGA
ACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTCTG
GAGGAGGAGGATCTTATTCAGAAGAGGAGTGTAAACAATACAAAGCAGTGGTC
TACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGGAGGTTG
AAGATAGACTTTGGTGACTCAGCCCGGGCGGATGATGCACGCCAACTCTTTGTG
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AAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGTTTCAACAGATCCCGAGA
GTACCAGCTTAATGATTCTGCAGCATACTATTTGAATGACTTGGACAGAATAGC
TCAACCAAATTACATCCCGACTCAACAAGATGTTCTCAGAACTAGAGTGAAAAC
TACAGGAATTGTTGAAACCCATTTTACTTTCAAAGATCTTCATTTTAAAATGTTT
GATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGGATTCATTGCTTCGAAGG
AGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGACTACGACCTGGTTCTAGC
TGAAGATGAAGAAATGAACCGAATGCATGAAAGCATGAAATTGTTTGACAGCA

TATGTAACAACAAGTGGTTTACAGATACATCCATTATACTTTTTCTAAACAAGA
AGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCTCACTATATGCTATCAAG
AATATGCAGGATCAAACACATATGAAGAGGCAGCTGCATATATTCAATGTCAGT
TTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAATATACACCCACTTCACA
TGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGATGCTGTAACAGATGTCA
TCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTAG

Coding Sequence of Gαi1(60)-mEGFP: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and green sequence represents mEGFP sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTCTGGAGGAGGAGGATCTATGGTGAGCAAGGGC
GAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTA
AACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGG
CAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCC
CACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGA
CCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCA
GGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGG
TGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGAC
TTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAG
CCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACT
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AGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACC
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TACAAGTCTGGAGGAGGAGGATCTTATTCAGAAGAGGAGTGTAAACAATACAA
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AACTCTTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCAGAACTTG

CTGGAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGTTTCAACA
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ACAGAATAGCTCAACCAAATTACATCCCGACTCAACAAGATGTTCTCAGAACTA
GAGTGAAAACTACAGGAATTGTTGAAACCCATTTTACTTTCAAAGATCTTCATTT
TAAAATGTTTGATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGGATTCATTG
CTTCGAAGGAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGACTACGACCT
GGTTCTAGCTGAAGATGAAGAAATGAACCGAATGCATGAAAGCATGAAATTGT
TTGACAGCATATGTAACAACAAGTGGTTTACAGATACATCCATTATACTTTTTCT
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ATGTCAGTTTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAATATACACCC
ACTTCACATGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGATGCTGTAAC
AGATGTCATCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTAG

Coding Sequence of Gαi1(91)-mEGFP: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and green sequence represents mEGFP sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTATTCAGAAGAGGAGTGTAAACAATACAAAGCA
GTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGG
AGGTTGTCTGGAGGAGGAGGATCTATGGTGAGCAAGGGCGAGGAGCTGTTCAC
CGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTT
CAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGA
AGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCA
CCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGC
ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCT
TCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGC
GACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGG
CAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATAT

CATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACA
ACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCC
ATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCC
AAGCTTAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTC
GTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCTGGAGGA
GGAGGATCTAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGATGCACGCCA
ACTCTTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCAGAACTTGC
TGGAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGTTTCAACAG
ATCCCGAGAGTACCAGCTTAATGATTCTGCAGCATACTATTTGAATGACTTGGA
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ATCAAGAATATGCAGGATCAAACACATATGAAGAGGCAGCTGCATATATTCAAT
GTCAGTTTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAATATACACCCAC
TTCACATGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGATGCTGTAACAG
ATGTCATCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTAG

Coding Sequence of Gαi1(91)-mCherry: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and red sequence represents mCherry sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTATTCAGAAGAGGAGTGTAAACAATACAAAGCA
GTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGG
AGGTTGTCTGGAGGAGGAGGATCTATGGTGAGCAAGGGCGAGGAGGATAACAT
GGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAA

CGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCA
CCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGG
ACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCG
CCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGC
GCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCC
TGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCT
CCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAG
CGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAA
GCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCA
AGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCA
CCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGC
CGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTCTGGAGGAGGAGGATC
TAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGATGCACGCCAACTCTTTGT
GCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCAGAACTTGCTGGAGTTAT
AAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGTTTCAACAGATCCCGAG
AGTACCAGCTTAATGATTCTGCAGCATACTATTTGAATGACTTGGACAGAATAG
CTCAACCAAATTACATCCCGACTCAACAAGATGTTCTCAGAACTAGAGTGAAAA
CTACAGGAATTGTTGAAACCCATTTTACTTTCAAAGATCTTCATTTTAAAATGTT
TGATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGGATTCATTGCTTCGAAG
GAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGACTACGACCTGGTTCTAG
CTGAAGATGAAGAAATGAACCGAATGCATGAAAGCATGAAATTGTTTGACAGC
ATATGTAACAACAAGTGGTTTACAGATACATCCATTATACTTTTTCTAAACAAG
AAGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCTCACTATATGCTATCAA
GAATATGCAGGATCAAACACATATGAAGAGGCAGCTGCATATATTCAATGTCAG
TTTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAATATACACCCACTTCAC
ATGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGATGCTGTAACAGATGTC
ATCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTAG

Coding Sequence of Gαi1(121)-mEGFP: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and green sequence represents mEGFP sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTATTCAGAAGAGGAGTGTAAACAATACAAAGCA
GTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGG
AGGTTGAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGATGCACGCCAACTC
TTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCATCTGGAGGAGGA
GGATCTATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTG
GTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGG
CGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGG
CAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCA
GTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGC
CATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAA
CTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCA
TCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAG
CTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAA
GAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCG
TGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGC
TGCTGCCCGACAACCACTACCTGAGCACCCAGTCCAAGCTTAGCAAAGACCCCA
ACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCA
CTCTCGGCATGGACGAGCTGTACAAGTCTGGAGGAGGAGGATCTGAACTTGCTG
GAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGTTTCAACAGAT
CCCGAGAGTACCAGCTTAATGATTCTGCAGCATACTATTTGAATGACTTGGACA
GAATAGCTCAACCAAATTACATCCCGACTCAACAAGATGTTCTCAGAACTAGAG
TGAAAACTACAGGAATTGTTGAAACCCATTTTACTTTCAAAGATCTTCATTTTAA
AATGTTTGATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGGATTCATTGCTT
CGAAGGAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGACTACGACCTGGT
TCTAGCTGAAGATGAAGAAATGAACCGAATGCATGAAAGCATGAAATTGTTTG

ACAGCATATGTAACAACAAGTGGTTTACAGATACATCCATTATACTTTTTCTAAA
CAAGAAGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCTCACTATATGCTA
TCAAGAATATGCAGGATCAAACACATATGAAGAGGCAGCTGCATATATTCAATG
TCAGTTTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAATATACACCCACT
TCACATGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGATGCTGTAACAG
ATGTCATCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTAG

Coding Sequence of Gαi1(121)-mCherry: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and red sequence represents mCherry sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTATTCAGAAGAGGAGTGTAAACAATACAAAGCA
GTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGG
AGGTTGAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGATGCACGCCAACTC
TTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCATCTGGAGGAGGA
GGATCTATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTT
CATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGAT
CGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGA
AGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGT
TCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACT
TGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGG
ACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCA
TCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGC
AGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGAC
GGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCA
CTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGC
CCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACT
ACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGC
ATGGACGAGCTGTACAAGTCTGGAGGAGGAGGATCTGAACTTGCTGGAGTTATA

AAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGTTTCAACAGATCCCGAGA
GTACCAGCTTAATGATTCTGCAGCATACTATTTGAATGACTTGGACAGAATAGC
TCAACCAAATTACATCCCGACTCAACAAGATGTTCTCAGAACTAGAGTGAAAAC
TACAGGAATTGTTGAAACCCATTTTACTTTCAAAGATCTTCATTTTAAAATGTTT
GATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGGATTCATTGCTTCGAAGG
AGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGACTACGACCTGGTTCTAGC
TGAAGATGAAGAAATGAACCGAATGCATGAAAGCATGAAATTGTTTGACAGCA
TATGTAACAACAAGTGGTTTACAGATACATCCATTATACTTTTTCTAAACAAGA
AGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCTCACTATATGCTATCAAG
AATATGCAGGATCAAACACATATGAAGAGGCAGCTGCATATATTCAATGTCAGT
TTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAATATACACCCACTTCACA
TGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGATGCTGTAACAGATGTCA
TCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTAG

Coding Sequence of Gαi1(60)-N-EGFP: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and green sequence represents N-EGFP sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTATTCAGAAGAGGAGTGTAAACAATACAAAGCA
GTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGG
AGGTTGTCTGGAGGAGGAGGATCTATGGTGAGCAAGGGCGAGGAGCTGTTCAC
CGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTT
CAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGA
AGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCA
CCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGC
ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCT
TCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGC
GACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGG
CAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATAT

CATGGCCGACAAGCAGTCTGGAGGAGGAGGATCTAAGATAGACTTTGGTGACT
CAGCCCGGGCGGATGATGCACGCCAACTCTTTGTGCTAGCTGGAGCTGCTGAAG
AAGGCTTTATGACTGCAGAACTTGCTGGAGTTATAAAGAGATTGTGGAAAGATA
GTGGTGTACAAGCCTGTTTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTG
CAGCATACTATTTGAATGACTTGGACAGAATAGCTCAACCAAATTACATCCCGA
CTCAACAAGATGTTCTCAGAACTAGAGTGAAAACTACAGGAATTGTTGAAACCC
ATTTTACTTTCAAAGATCTTCATTTTAAAATGTTTGATGTGGGAGGTCAGAGATC
TGAGCGGAAGAAGTGGATTCATTGCTTCGAAGGAGTGGCGGCGATCATCTTCTG
TGTAGCACTGAGTGACTACGACCTGGTTCTAGCTGAAGATGAAGAAATGAACCG
AATGCATGAAAGCATGAAATTGTTTGACAGCATATGTAACAACAAGTGGTTTAC
AGATACATCCATTATACTTTTTCTAAACAAGAAGGATCTTTTTGAAGAAAAAAT
CAAAAAGAGCCCTCTCACTATATGCTATCAAGAATATGCAGGATCAAACACATA
TGAAGAGGCAGCTGCATATATTCAATGTCAGTTTGAAGACCTCAATAAAAGAAA
GGACACAAAGGAAATATACACCCACTTCACATGTGCCACAGATACTAAGAATGT
GCAGTTTGTTTTTGATGCTGTAACAGATGTCATCATAAAAAATAATCTAAAAGA
TTGTGGTCTCTTTTAG

Coding Sequence of Gαi1(60)-C-EGFP: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and green sequence represents C-EGFP sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTATTCAGAAGAGGAGTGTAAACAATACAAAGCA
GTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGG
AGGTTGTCTGGAGGAGGAGGATCTAAGAACGGCATCAAGGTGAACTTCAAGAT
CCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGA
ACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCA
CCCAGTCCAAGCTTAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGC
TGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGT
CTGGAGGAGGAGGATCTAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGAT

GCACGCCAACTCTTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCA
GAACTTGCTGGAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGT
TTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTGCAGCATACTATTTGAAT
GACTTGGACAGAATAGCTCAACCAAATTACATCCCGACTCAACAAGATGTTCTC
AGAACTAGAGTGAAAACTACAGGAATTGTTGAAACCCATTTTACTTTCAAAGAT
CTTCATTTTAAAATGTTTGATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGG
ATTCATTGCTTCGAAGGAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGAC
TACGACCTGGTTCTAGCTGAAGATGAAGAAATGAACCGAATGCATGAAAGCAT
GAAATTGTTTGACAGCATATGTAACAACAAGTGGTTTACAGATACATCCATTAT
ACTTTTTCTAAACAAGAAGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCT
CACTATATGCTATCAAGAATATGCAGGATCAAACACATATGAAGAGGCAGCTGC
ATATATTCAATGTCAGTTTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAA
TATACACCCACTTCACATGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGA
TGCTGTAACAGATGTCATCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTA

Coding Sequence of Gαi1(91)-N-EGFP: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and green sequence represents N-EGFP sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTATTCAGAAGAGGAGTGTAAACAATACAAAGCA
GTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGG
AGGTTGAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGATGCACGCCAACTC
TTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCATCTGGAGGAGGA
GGATCTATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTG
GTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGG
CGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGG
CAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCA
GTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGC
CATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAA

CTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCA
TCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAG
CTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGTCT
GGAGGAGGAGGATCTGAACTTGCTGGAGTTATAAAGAGATTGTGGAAAGATAG
TGGTGTACAAGCCTGTTTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTGC
AGCATACTATTTGAATGACTTGGACAGAATAGCTCAACCAAATTACATCCCGAC
TCAACAAGATGTTCTCAGAACTAGAGTGAAAACTACAGGAATTGTTGAAACCCA
TTTTACTTTCAAAGATCTTCATTTTAAAATGTTTGATGTGGGAGGTCAGAGATCT
GAGCGGAAGAAGTGGATTCATTGCTTCGAAGGAGTGGCGGCGATCATCTTCTGT
GTAGCACTGAGTGACTACGACCTGGTTCTAGCTGAAGATGAAGAAATGAACCG
AATGCATGAAAGCATGAAATTGTTTGACAGCATATGTAACAACAAGTGGTTTAC
AGATACATCCATTATACTTTTTCTAAACAAGAAGGATCTTTTTGAAGAAAAAAT
CAAAAAGAGCCCTCTCACTATATGCTATCAAGAATATGCAGGATCAAACACATA
TGAAGAGGCAGCTGCATATATTCAATGTCAGTTTGAAGACCTCAATAAAAGAAA
GGACACAAAGGAAATATACACCCACTTCACATGTGCCACAGATACTAAGAATGT
GCAGTTTGTTTTTGATGCTGTAACAGATGTCATCATAAAAAATAATCTAAAAGA
TTGTGGTCTCTTTTAG

Coding Sequence of Gαi1(91)-C-EGFP: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and green sequence represents C-EGFP sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTATTCAGAAGAGGAGTGTAAACAATACAAAGCA
GTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGG
AGGTTGAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGATGCACGCCAACTC
TTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCATCTGGAGGAGGA
GGATCTAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGA
CGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACG
GCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCAAGCTTAGCA

AAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCG
CCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCTGGAGGAGGAGGATCT
GAACTTGCTGGAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGT
TTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTGCAGCATACTATTTGAAT
GACTTGGACAGAATAGCTCAACCAAATTACATCCCGACTCAACAAGATGTTCTC
AGAACTAGAGTGAAAACTACAGGAATTGTTGAAACCCATTTTACTTTCAAAGAT
CTTCATTTTAAAATGTTTGATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGG
ATTCATTGCTTCGAAGGAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGAC
TACGACCTGGTTCTAGCTGAAGATGAAGAAATGAACCGAATGCATGAAAGCAT
GAAATTGTTTGACAGCATATGTAACAACAAGTGGTTTACAGATACATCCATTAT
ACTTTTTCTAAACAAGAAGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCT
CACTATATGCTATCAAGAATATGCAGGATCAAACACATATGAAGAGGCAGCTGC
ATATATTCAATGTCAGTTTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAA
TATACACCCACTTCACATGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGA
TGCTGTAACAGATGTCATCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTA

Coding Sequence of Gαi1(121)-N-EGFP: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and green sequence represents N-EGFP sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTCTGGAGGAGGAGGATCTAAGAACGGCATCAAG
GTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGA
CCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAA
CCACTACCTGAGCACCCAGTCCAAGCTTAGCAAAGACCCCAACGAGAAGCGCG
ATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGG
ACGAGCTGTACAAGTCTGGAGGAGGAGGATCTTATTCAGAAGAGGAGTGTAAA
CAATACAAAGCAGTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATT
AGGGCTATGGGGAGGTTGAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGA
TGCACGCCAACTCTTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGC

AGAACTTGCTGGAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTG
TTTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTGCAGCATACTATTTGAAT
GACTTGGACAGAATAGCTCAACCAAATTACATCCCGACTCAACAAGATGTTCTC
AGAACTAGAGTGAAAACTACAGGAATTGTTGAAACCCATTTTACTTTCAAAGAT
CTTCATTTTAAAATGTTTGATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGG
ATTCATTGCTTCGAAGGAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGAC
TACGACCTGGTTCTAGCTGAAGATGAAGAAATGAACCGAATGCATGAAAGCAT
GAAATTGTTTGACAGCATATGTAACAACAAGTGGTTTACAGATACATCCATTAT
ACTTTTTCTAAACAAGAAGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCT
CACTATATGCTATCAAGAATATGCAGGATCAAACACATATGAAGAGGCAGCTGC
ATATATTCAATGTCAGTTTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAA
TATACACCCACTTCACATGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGA
TGCTGTAACAGATGTCATCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTA
G

Coding Sequence of Gαi1(91)-C-EGFP: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and green sequence represents C-EGFP sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTATTCAGAAGAGGAGTGTAAACAATACAAAGCA
GTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATTAGGGCTATGGGG
AGGTTGAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGATGCACGCCAACTC
TTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCATCTGGAGGAGGA
GGATCTAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGA
CGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACG
GCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCAAGCTTAGCA
AAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCG
CCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCTGGAGGAGGAGGATCT
GAACTTGCTGGAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGT

TTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTGCAGCATACTATTTGAAT
GACTTGGACAGAATAGCTCAACCAAATTACATCCCGACTCAACAAGATGTTCTC
AGAACTAGAGTGAAAACTACAGGAATTGTTGAAACCCATTTTACTTTCAAAGAT
CTTCATTTTAAAATGTTTGATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGG
ATTCATTGCTTCGAAGGAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGAC
TACGACCTGGTTCTAGCTGAAGATGAAGAAATGAACCGAATGCATGAAAGCAT
GAAATTGTTTGACAGCATATGTAACAACAAGTGGTTTACAGATACATCCATTAT
ACTTTTTCTAAACAAGAAGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCT
CACTATATGCTATCAAGAATATGCAGGATCAAACACATATGAAGAGGCAGCTGC
ATATATTCAATGTCAGTTTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAA
TATACACCCACTTCACATGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGA
TGCTGTAACAGATGTCATCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTA
G

Coding Sequence of Gαi1(121)-C-EGFP: Black sequence corresponds to Gαi1 protein gene; blue sequence shows linker sequences and green sequence represents C-EGFP sequence.

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGTAAGAT
GATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGCGAGGTCAAGC
TGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACAATTGTGAAGCAGATGA
AAATTATCCATGAAGCTGGTTCTGGAGGAGGAGGATCTAAGAACGGCATCAAG
GTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGA
CCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAA
CCACTACCTGAGCACCCAGTCCAAGCTTAGCAAAGACCCCAACGAGAAGCGCG
ATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGG
ACGAGCTGTACAAGTCTGGAGGAGGAGGATCTTATTCAGAAGAGGAGTGTAAA
CAATACAAAGCAGTGGTCTACAGTAACACCATCCAGTCAATTATTGCTATCATT
AGGGCTATGGGGAGGTTGAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGA
TGCACGCCAACTCTTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGC
AGAACTTGCTGGAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTG
TTTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTGCAGCATACTATTTGAAT

GACTTGGACAGAATAGCTCAACCAAATTACATCCCGACTCAACAAGATGTTCTC
AGAACTAGAGTGAAAACTACAGGAATTGTTGAAACCCATTTTACTTTCAAAGAT
CTTCATTTTAAAATGTTTGATGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGG
ATTCATTGCTTCGAAGGAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGAC
TACGACCTGGTTCTAGCTGAAGATGAAGAAATGAACCGAATGCATGAAAGCAT
GAAATTGTTTGACAGCATATGTAACAACAAGTGGTTTACAGATACATCCATTAT
ACTTTTTCTAAACAAGAAGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCT
CACTATATGCTATCAAGAATATGCAGGATCAAACACATATGAAGAGGCAGCTGC
ATATATTCAATGTCAGTTTGAAGACCTCAATAAAAGAAAGGACACAAAGGAAA
TATACACCCACTTCACATGTGCCACAGATACTAAGAATGTGCAGTTTGTTTTTGA
TGCTGTAACAGATGTCATCATAAAAAATAATCTAAAAGATTGTGGTCTCTTTTA
G

E. PixFRET ANALYSIS

Table E.1 The Percantage of Every Range of Gαi1 Homodimerization by PixFRET

Table Analyzed	Gi-Gi+5x Gi-wt 1-10	Gi-Gi+5x Gi-wt 11-20	Gi-Gi+5x Gi-wt 21-30	Gi-Gi+5x Gi-wt 31-40	Gi-Gi+5x Gi-wt 41-50
Unpaired t test					
P value	<0,0001	0,0359	<0,0001	<0,0001	<0,0001
P value summary	****	*	****	****	****
Significantly different (P < 0.05)?	Yes	Yes	Yes	Yes	Yes
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed	Two-tailed	Two-tailed
t, df	t=4,739 df=61	t=2,14 df=69	t=5,595 df=50	t=6,061 df=47	t=8,954 df=46
How big is the difference?					
Mean ± SEM of column A	46,64 ± 1,686, n=20	28,23 ± 0,339, n=21	14,56 ± 0,6767, n=21	6,911 ± 0,6494, n=21	3,744 ± 0,4494, n=17
Mean ± SEM of column B	54,63 ± 0,8446, n=43	27,21 ± 0,2735, n=50	10,85 ± 0,298, n=31	3,392 ± 0,1312, n=28	0,6633 ± 0,07214, n=31
Difference between means	7,993 ± 1,686	-1,019 ± 0,4763	-3,708 ± 0,6627	-3,519 ± 0,5806	-3,08 ± 0,344
95% confidence interval	4,621 to 11,37	-1,97 to -0,0691	-5,039 to -2,377	-4,687 to -2,351	-3,773 to -2,388

F. FRET EFFICIENCY WITH MICROPLATE READER

Table F.1 :Pairwise t-test analysis of the FRET efficiency data with microplate reader.

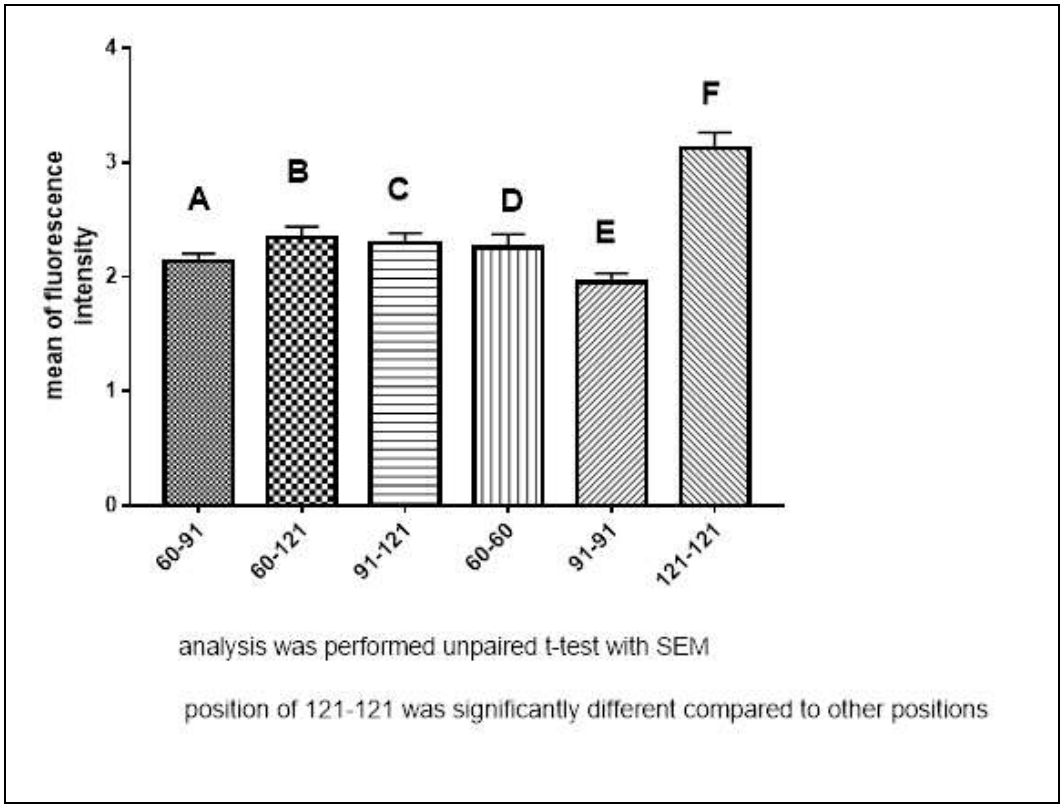
Unpaired t test data analysis	Gαi1-Gαi1 + 5X Gαi1-wt & Gαi1 -Gαi1 FRET	soluble EGFP + mCherry & Gαi1-Gαi1 + 5X Gαi1-wt	Gαi1-Gαi1 + 5X Gαi1-wt & Gαi1-EGFP +membrane targeted mch FRET	Gαi1 -Gαi1 FRET & soluble EGFP + mCherry FRET	Gαi1 -Gαi1 FRET & Gαi1-EGFP +membrane targeted mch FRET
P value	0,0592	<0,0001	0,0743	0,0002	0,0425
P value summary	ns	****	ns	***	*
Significantly different (P < 0.05)?	No	Yes	No	Yes	Yes
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed	Two-tailed	Two-tailed
t, df	t=3,926 df=2	t=28,92 df=3	t=3,46 df=2	t=21,38 df=3	t=4,696 df=2
Mean ± SEM of column A	0,8385 ± 0,0354, n=2	0,6976 ± 0,005947, n=2	0,6976 ± 0,005947, n=2	0,8385 ± 0,0354, n=2	0,8385 ± 0,0354, n=2
Mean ± SEM of column B	0,6976 ± 0,005947, n=2	0,1515 ± 0,01422, n=3	0,3899 ± 0,08874, n=2	0,1515 ± 0,01422, n=3	0,3899 ± 0,08874, n=2
Difference between means	-0,1409 ± 0,03589	-0,5461 ± 0,01888	-0,3077 ± 0,08894	-0,687 ± 0,03214	-0,4487 ± 0,09554
95% confidence interval	-0,2953 to 0,01353	-0,6062 to -0,486	-0,6904 to 0,07494	-0,7893 to -0,5847	-0,8597 to -0,03757
R squared (eta squared)	0,8851	0,9964	0,8569	0,9935	0,9168

G.LUMINESCENCE VALUES OF WILD-TYPE GAI1 GENES WITH CAMP-GLO ASSAY

Table G.1: Measurement results of the luminescence of wild-type Gαi1 genes

The only forskolin applied wild-type Gαi1			Forskolin + quinpirole applied wild-type Gαi1		
Well 1	Well 2	Well 3	Well 1	Well 2	Well 3
1922000	582800	955400	1462000	1368000	1407000

H. FLUORESCENCE INTENSITIES OF FRET STUDIES



	P value
A-F	<0,0001
B-F	<0,0001
C-F	<0,0001
D-F	<0,0001
E-F	<0,0001