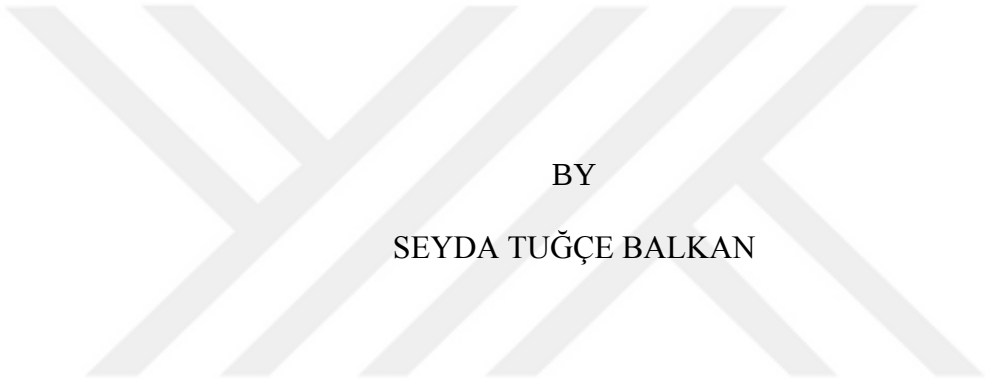


INVESTIGATION OF PHYSICAL INTERACTION BETWEEN Gαi AND Gαs
PROTEINS VIA FRET IN LIVE CELLS

A THESIS SUBMITTED TO
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Approval of the thesis:

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Gas PROTEINS VIA FRET IN LIVE CELLS**

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ABSTRACT

INVESTIGATION OF PHYSICAL INTERACTION BETWEEN *G_{ai}* AND *G_{as}* PROTEINS via FRET IN LIVE CELLS

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August 2021, 111 pages

GPCR's are seven-transmembrane receptors that transmit external signals to the intracellular environment via secondary messenger systems through heterotrimeric G proteins. Heterotrimeric G proteins consist of α and β - γ subunits. Until recent years, scientists thought that GPCR signal transduction occurs between one GPCR and one heterotrimeric G protein; however, recently, it has been shown that GPCR's can make oligomers. Oligomerization of GPCR allows cells to tune the intensity of the signal and respond appropriately. Studies with A1 and A2A heterotetramer and A2A – D2R heterotetramer showed that these interactions theoretically allow G protein α subunit dimer formation according to Navarro *et al.* and Ferré *et al.* This theoretical interaction has never been validated via an advanced fluorescent microscopy technique like FRET. This technique allows the detection of physical protein-protein interaction of fluorescently labeled proteins via resonance energy transfer between donor and acceptor fluorophores. In this study, *G_{ai}* and *G_{as}* interaction was investigated by FRET technique. In addition, the effect of agonist treatment, CGS (CGS-21680), and quinpirole for A2A and D2R heterotetramer,

respectively, was investigated. Furthermore, based on the G protein family member K-Ras studies, which has high structural homology with G protein α subunits, that forms a homodimer independent from its receptor (Dempke & Heinemann, 2009), we tested the effect of receptor binding on the physical interaction of G protein α subunit using G protein α subunit-specific minigenes.

Keywords: G α Proteins, Protein Interactions, FRET, Dimerization, Receptor Dependency



ÖZ

Gai VE Gas PROTEİNLERİNİN FİZİKSEL ETKİLEŞİMLERİNİN CANLI HÜCRELERDE FRET METODU İLE ARAŞTIRILMASI

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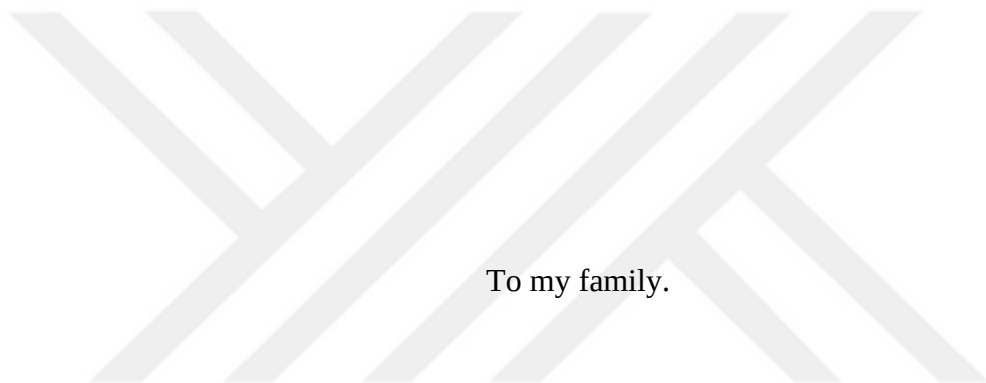
Ağustos 2021, 111 sayfa

Hücre zarını yedi defa geçen hücre zarı protein ailesi GPCR'ler, hücre dışı sinyalleri hücre içindeki ikincil haberci yoluna heterotrimerik G proteinleri ile iletirler. Heterotrimerik G proteinleri α ve β - γ altbirimlerinden oluşur. Geleneksel olarak bir GPCR'nin bir heterotrimerik G protein ile etkileştiği düşünülüyordu, ancak son yıllardaki çalışmalar GPCR oligomerizasyonunu göstermektedir. GPCR oligomerizasyonu, hücrelerin sinyal iletimi dengesini ve gereken cevabı vermesini sağlamaktadır. Navarro ve arkadaşlarının ve Ferré ve arkadaşlarının A1 ve A2A reseptör heterotetramerleri ve A2A ve D2 reseptör heterotetramerleri ile yaptıkları çalışmalar bu yapılarda teorik olarak G protein α altbirimlerinin etkileşim yakınlığında bulunduğunu göstermektedir. Bu teorik etkileşim, daha önce, FRET gibi ileri floresan mikroskopi teknikleri kullanılarak valide edilmemiştir. FRET tekniği donör florofor ve akseptör florofor ile işaretlenen proteinlerin, donör ve akseptör proteinler arasında gerçekleşen enerji transferi vasıtası ile fiziksel protein-protein etkileşimlerinin incelenmesine izin vermektedir. Bu tez çalışmasında, Gai ve Gas etkileşimleri FRET tekniği ile araştırılmıştır. Ek olarak agonist varlığında, CGS (CGS-21680) ve Quinpirole kullanılarak A2A ve D2 reseptör heterotetramer etkisi araştırılmıştır. G protein ailesi üyesi ve G protein α altbirimi ile yüksek yapısal

homolojisi olan K-Ras proteininin reseptör bağımsız homodimerizasyon çalışmalarına dayanılarak (Dempke & Heinemann, 2009), G α proteinlerinin reseptör etkileşimlerinin fiziksel etkileşimlerine olan etkisi, G α spesifik minigenler kullanılarak araştırılmıştır.

Anahtar Kelimeler: G α Proteini, Protein Etkileşimleri, FRET, Dimerizasyon, Receptor Bağımlılığı





To my family.

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“If one day, my words are against science, choose science.”

“Science is the most reliable guide for civilization, for life, for success in the world. Searching a guide other than science means carelessness, ignorance and heresy.”

“Heroic Turkish woman, you are worthy not to crawl on the ground, but to rise to the sky on your shoulders.”

–Mustafa Kemal Atatürk

TABLE OF CONTENTS

ABSTRACT	v
ÖZ.....	vii
ACKNOWLEDGMENTS.....	x
TABLE OF CONTENTS	xii
LIST OF TABLES	xvi
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xxi
INTRODUCTION.....	1
1.1 GTP Binding Proteins.....	1
1.1.1 Small GTPases.....	1
1.1.2 Heterotrimeric G-proteins	2
1.1.2.1 G α subunits:.....	4
1.2 GPCR signaling.....	7
1.2	8
1.2.1 Classical GPCR Interaction Model	8
1.2.2 GPCR oligomerization	8
1.3 G-protein signaling.....	9
1.3.1 Signaling of Small GTPases	10
1.3.2 Signaling of Heterotrimeric G protein.....	11
1.4 Protein-Protein Interactions	12
1.4.1 Protein-protein Interaction detection Methods in vivo	13

1.4.1.1 Förster Resonance Energy Transfer (FRET)	14
1.4.1.1.1 Confocal microscopy fluorescent imaging	18
1.4.1.1.2 Monochromator Plate Reader	20
1.3.1	21
1.4.1.2 Minigenes.....	21
1.5 Aim of Study	22
MATERIALS AND METHODS.....	25
2.1 Materials.....	25
2.1.1 Mammalian Cell Culture.....	25
2.1.2 Bacteria culture	26
2.1.3 Cloning.....	27
2.2 Methods.....	28
2.2.1 Cloning.....	28
2.2.2 Mammalian Cell culture	35
2.2.3 Fluorescence Measurements	37
2.2.4 Image Analysis with Pix-FRET Program	39
2.2.5 Functional Analysis with cAMP-Glo™ Assay	40
2.2.6 Statistical analysis	42
RESULTS AND DISCUSSION	43
3.3 Investigation of Interaction Between G α i and G α s Proteins with FRET by Using Confocal Microscope.....	48
3.4 Investigation of G α i and G α s Interaction with FRET Technique Using Fluorescence Plate Reader	53
3.5 Preparation and Sequence Analysis of G α i and G α s Specific Minigenes	56

3.6	Investigation of the Receptor Dependency of G α i and G α s Interaction by Using Minigenes.....	59
3.6.1	Investigation by Using Confocal Microscopy	59
3.6.2	Investigation by Using Fluorescence Plate Reader	64
3.7	Investigation of G α i and G α s Interaction with Agonist Treatment for D2R and A2R Receptor Signaling Pathway Activation by Using Fluorescence Plate Reader.....	68
3.8	Future Studies	72
3.9	Discussion.....	73
	CONCLUSION	75
	REFERENCES	77
A.	COMPOSITIONS OF SOLUTIONS	86
B.	MAMMALIAN EXPRESSION MAPS	90
C.	DESIGNED PRIMERS	91
D.	FUSION PROTEIN CODING SEQUENCES	92
E.	cAMP GLO ASSAY STATISTICAL ANALYSIS	105
F.	STATISTICAL ANALYSIS OF THE CONFOCAL MICROSCOPY RESULTS Of G α i-G α s FRET PAIRS	106
G.	MONOCHROMATOR PLATE READER RESULT STATISTICAL ANALYSYS OF G α i-G α s FRET PAIRS.....	107
H.	STATISTICAL ANALYSIS OF THE CONFOCAL MICROSCOPY RESULTS Of G α i-G α s FRET PAIRS WITH G α i and G α s PROTEIN SPECIFIC MINIGENES	108
I.	MONOCHROMATOR PLATE READER RESULT STATISTICAL ANALYSYS OF G α i-G α s FRET PAIRS WITH G α i and G α s PROTEIN SPECIFIC MINIGENES	109

J.	WITH/WITHOUT CGS+QUINPIROLE TREATMENT RESULT ANALYSIS OF $G\alpha_i$ - $G\alpha_s$ FRET GROUP WITH/WITHOUT MINIGENE	110
K.	CURVE FITTING STATISTICAL ANALYSIS	111



LIST OF TABLES

TABLES

Table 2. 1: Optimal PCR conditions for Gα tagging	29
Table 2. 2: Optimal PCR conditions for Gαi Minigene Preparation	30
Table 2. 3: Optimal PCR conditions for Gαs Minigene Preparation.....	30
Table 2. 4: PCR mixture for Gα minigene Preparation	30
Table 2. 5: Optimized integration PCR protocol.....	31
Table A. 1: DMEM High Glucose Compomposition.....	86
Table A. 2: Composition of TFB I Solution	89
Table A. 3: Composition of TFB II Solution.....	89
Table C. 1: Minigene Cloning Primers	91

LIST OF FIGURES

FIGURES

Figure 1. 1: G α protein family members, expression patterns and % amino acid identities with each other	3
Figure 1. 2: G β and G γ protein family members , expression patterns and % amino acid identities with each other.....	4
Figure 1. 3: G α protein molecular structure and domains	4
Figure 1. 4: G α i protein structure.....	6
Figure 1. 5: Schematic representation of Classical model of GPCR and heterotrimeric G protein interaction.....	8
Figure 1. 6: Small G protein signal transduction.	10
Figure 1. 7: K-Ras signaling pathways.	11
Figure 1. 8: A1 and A2A receptor heterotetrameric model coupling with G α i and G α s proteins.	12
Figure 1. 9: Protein-protein interaction types.....	13
Figure 1. 10: EGFP and mCherry fluorophore excitation and emission spectrum	17
Figure 1. 11: Imaging principles of A)widefield epifluorescence and B)Confocal microscope	19
Figure 1. 12: Spinning disc microscope component schematic representation. ..	20
Figure 1. 13: Monochromator plate reader component schematic representation.	21
Figure 1. 14: G protein alpha subunit specific minigenes and sequence domains.	22
Figure 2. 1: Overlapping extension PCR representation.	32
Figure 2. 2: cAMP-Glo™ Assay kit working principle.	41
Figure 3. 1: cAMP-Glo Assay results of G α proteins labeled with EGFP and mCherry fluorophores.	43
Figure 3. 2: Membrane targeted organelle marker Gap43 sequence labeled with A) mCherry and B) EGFP.....	46
Figure 3. 3: Fluorescent protein labeled G α s Δ 73-85 protein visualization.	47
Figure 3. 4: Fluorescent protein labeled G α i 91 protein visualization.....	47

Figure 3. 5: EGFP Fluorescent protein labeled Gai 121 protein visualization..	48
Figure 3. 6: Confocal FRET imaging of Gai(91)-EGFP and Gas(73-85)-mCherry..	50
Figure 3. 7: Confocal FRET imaging of Gai(121)-EGFP and Gas(73-85)-mCherry.	51
Figure 3. 8: Confocal FRET imaging of Gap43 EGFP and Gap43-mCherry..	52
Figure 3. 9: Confocal FRET imaging of Gap43mCherry-L-EGFP. 300ng of plasmid transfected into N2a cell line..	52
Figure 3. 10: Pix FRET analysis of confocal images.	53
Figure 3. 11: Spectral area normalization of FRET spectrum of the Gai and Gas FRET pairs.....	55
Figure 3. 12: Acceptor / Donor ratio of normalized spectrum of Gai-Gas and FRET control groups.	56
Figure 3. 13: Restriction sites for PstI double cutter for pcDNA 3.1(-).....	57
Figure 3. 14: Gai and Gas minigene cloning scheme.ö m56	58
Figure 3. 15: Gai minigene cloning agarose gel results.	58
Figure 3. 16: Gas minigene cloning agarose gel results.	59
Figure 3. 17: LSM Airy scan Confocal FRET imaging of Gai(91)-EGFP and Gas(73-85)-mCherry. 500ng of Gai(91)-EGFP and Gas(73-85)-mCherry co-transfected into N2a cell line.....	61
Figure 3. 18: LSM Airy scan Confocal FRET imaging of Gai(121)-EGFP and Gas(73-85)-mCherry..	62
Figure 3. 19: LSM Airy scan Confocal FRET imaging of Gai(121)-EGFP and Gas(73-85)-mCherry + Gai and Gas minigenes..	63
Figure 3. 20: Pix FRET analysis of confocal images.	64
Figure 3. 21: Spectral area normalization of FRET spectrum of the Gai-Gas and minigene added Gai-Gas FRET pairs.	66
Figure 3. 22: Acceptor / Donor ratio of normalized spectrum of Gai-Gas and FRET control groups	67

Figure 3. 23: Spectral area normalization of FRET spectrum of the Gai-Gas and minigene added Gai-Gas FRET pairs with and without CGS+Quinpirole treatment..	69
Figure 3. 24: Acceptor / Donor ratio of the normalized spectrum of Gai-Gas and FRET control groups with CGS and Quinpirole treatment.	71
Figure 3. 25: Location-dependent curve fitting results with MATLAB... Hata! Yer işareti tanımlanmamış.	
Figure B. 1: pcDNA3.1(-) expression vector map	90
Figure E. 1: Statistical analysis of Gai fusion protein functional assay results...	105
Figure F. 1: Ordinary One way ANOVA analysis of Gai(121) EGFP-Gas(73-85)mCherry , Gai(91) EGFP-Gas(73-85)mCherry and Gap43 EGFP+ Gap43 mCherry negative control group confocal microscope results calculated with Pix FRET	106
Figure G. 1: Gai(121) EGFP-Gas(73-85)mCherry , Gai(91) EGFP-Gas(73-85)mCherry and Gap43 FRET control groups mCherry spectral peak region result analysis with Ordinary One way ANOVA	107
Figure G. 2: Gai(121) EGFP-Gas(73-85)mCherry , Gai(91) EGFP-Gas(73-85)mCherry and Gap43 FRET control groups Acceptor/Donor peak area result analysis with Ordinary One way ANOVA	107
Figure H. 1: LSM Confocal microscope result from Gai(121) EGFP-Gas(73-85)mCherry , Gai(91) EGFP-Gas(73-85)mCherry and Gai(121) EGFP-Gas(73-85)mCherry with minigene FRET groups analyzed with Ordinary One Way Anova analysis	108
Figure I. 1: Monochromator plate reader results of Gai(121) EGFP-Gas(73-85)mCherry, Gai(91) EGFP-Gas(73-85)mCherry and Gai(121) EGFP-Gas(73-85)mCherry with minigene FRET groups Ordinary One Way Anova analysis	109

Figure I. 2: Acceptor/Donor spectral peak area results of G α i(121) EGFP-G α s(73-85)mCherry, G α i(91) EGFP-G α s(73-85)mCherry and G α i(121) EGFP-G α s(73-85)mCherry with minigene FRET groups Ordinary One Way Anova analysis.... 109

Figure J. 1: Ordinary One Way ANOVA analysis of mCherry spectrum peak normalized area comparison of G α i-G α s FRET pairs with/without G α protein specific minigenes and with/without CGS+Quinpirole treatment..... 110

Figure J. 2: One way ANOVA analysis of Acceptor/Donor spectral area normalized peak results of G α i-G α s FRET pairs with/without G α protein specific minigenes and with/without CGS+Quinpirole treatment. 110

Figure K. 1: Signal localization curve fit Two way ANOVA analysis results of FRET pairs. 111

Figure K. 2: Membrane curve fit result One Way ANOVA analysis of FRET pairs 111

LIST OF ABBREVIATIONS

ABBREVIATIONS

A2A	Adenosine 2A
AC	Adenylyl Cyclase
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
β_2 AR	Beta-2 Adrenergic Receptor
Barr2	Beta-Arrestin 2
bp	base pair
cAMP	cyclic AMP
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetraacetic acid
EE	Early Endosomes
FBS	Fetal Bovine Serum
FRET	Förster Resonance Energy Transfer
GFP	Green Fluorescent Protein
GPCR	G Protein Coupled Receptors

kb	kilo base pair
LB	Luria Bertani
MAPK	Mitogen-Activated Protein Kinase
mCherry	Monomeric Cherry
mEGFP	Monomeric Enhanced Green Fluorescent Protein
N2a	Neuro2a
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PPIs	Protein-protein interactions
SBT	Spectral bleed-through

CHAPTER 1

INTRODUCTION

1.1 GTP Binding Proteins

In multicellular organisms, homeostasis mainly depends on signal transduction, which allows the organism to respond properly to changes that originate from both the organism and the environment. Signal transduction in such organisms includes Guanine binding proteins (G-proteins) and G protein coupled receptors (GPCRs). G-protein family is an extensive family, and their members have evolutionarily conserved domains, which bind GTP/GDP and their receptors. GPCRs are one of the most abundant receptors on cell membrane. When a GPCR binds to its ligand, the G-protein is activated, then GDP-GTP exchange from G-protein transduces the extracellular signal to the intracellular response. G protein signal transduction is required for cellular responses, including cell growth, protein synthesis, and membrane vesicle transport. In general, G-proteins are classified under two main classes as small G proteins (small GTPases) and heterotrimeric G proteins.

1.1.1 Small GTPases

Members of small G-protein sub family are homologous of the alpha subunit of heterotrimeric G proteins. Small GTPases are key proteins in cellular signal transductions and cellular responses such as sustaining cell polarity, progressing cell cycle, and reorganizing cytoskeleton of the cell in eukaryotic cells. Considering their 3D structure, sequence, and function, despite containing over 150 members, small G

protein family have only five main groups: Ras, Rho, Arf/Sar, Rab, and Ran. Among those groups, Ras proteins are the most studied member of these groups (Song et al., 2019). H-Ras, K-Ras4A and 4B, and N-Ras are Ras family members and are responsible for regulating cell growth, division, differentiation, and death by utilizing signaling pathways (Repasky et al., 2010).

1.1.2 Heterotrimeric G-proteins

Apart from Small G proteins and Ras family, heterotrimeric G proteins were reported firstly by Alfred Gilman and Martin Rodbell 30 years ago. Members of heterotrimeric G proteins have three subunits; alpha, beta, and gamma. Alpha subunit of heterotrimeric G proteins has a binding affinity to GTP and GDP. Various subtypes of this subunit is present such as $G_{\alpha i}$, $G_{\alpha s}$, $G_{\alpha q}$ and $G_{\alpha 12}$, where each has different signal transduction paths. Similarly, beta-gamma subunits have many different sub-types, and these subunits act as a dimer that target different effectors. Together with alpha subunit, this family trigger many effectors that regulate ion channels, adenylyl cyclase, phosphoinositide-specific phospholipase C (PI-PLC) and phosphodiesterase (PDE) (Zachariou et al., 2012).

G_{α} protein family members in the human genome have 35-95% homology with each other. In addition, 23 types of G_{α} proteins are divided into four main subclasses: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$. $G_{\alpha s}$ family includes $G_{\alpha s}$ and $G_{\alpha olf}$. While $G_{\alpha s}$ expressed by many cells of the body, $G_{\alpha olf}$ expression is mainly from olfactory sensory neurons. $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o}$, $G_{\alpha it-rod}$, $G_{\alpha t-cone}$, $G_{\alpha igust}$ and $G_{\alpha iz}$ belong to $G_{\alpha i}$ family diagrams are presented in Figure 1.1. $G_{\alpha i}$ family is the most variegated family among others. The main effect of $G_{\alpha i}$ family, is the inhibition of the cAMP-dependent protein kinase (Boularan & Gales, 2015).

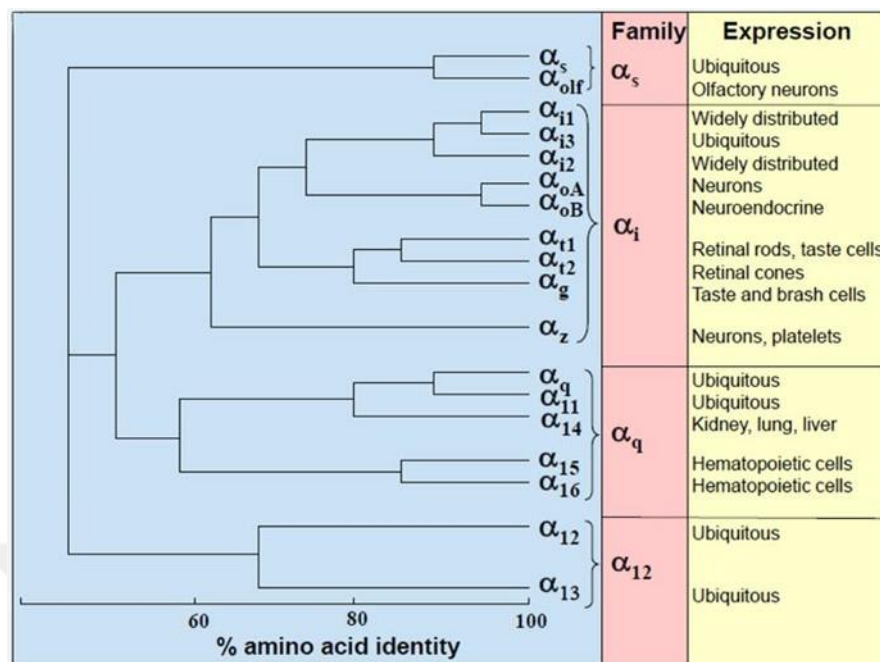


Figure 1. 1: G α protein family members, expression patterns and % amino acid identities with each other (Taken from (Syrovatkina et al., 2016)).

The beta-gamma dimer of heterotrimeric G proteins has a variety of members. G β family has five members that have homology around 80-90% with each other. G β 5 member of the family shows 50% sequence matching and is expressed at the brain cells. In addition, G β 1 expression is seen in retinal rods and G β 4 expression is seen in retinal cones while other members' expression location is widely distributed to all body cells. G γ family has 12 members and the members have 30-70% sequence identity as given in Figure 1.2 (Syrovatkina et al., 2016).

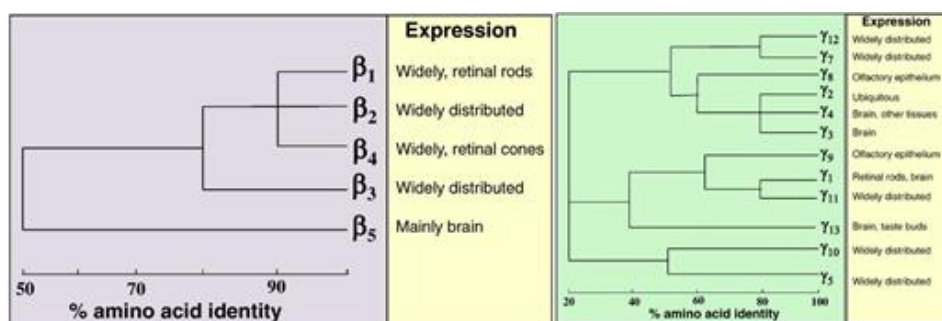


Figure 1. 2: G β and G γ protein family members , expression patterns and % amino acid identities with each other (Taken from (Syrovatkina et al., 2016)).

1.1.2.1 G α subunits:

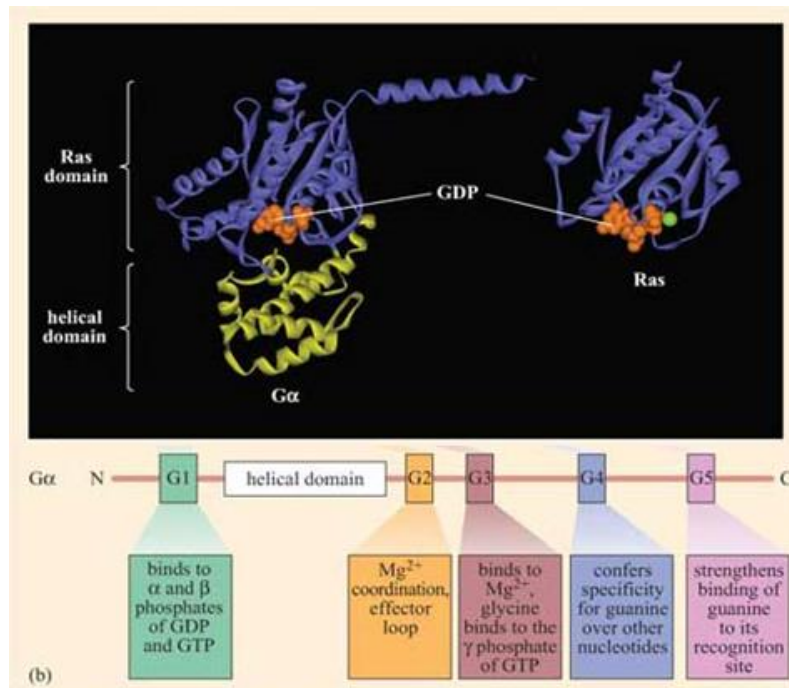


Figure 1. 3: G α protein molecular structure and domains. A) G α protein 3D molecular structure, B) G α protein domains and functionalities (Adapted from (*Cell Signalling: 3.5 Monomeric G Proteins* - OpenLearn - Open University - S377_4, n.d.)).

G α proteins consist of 2 main domains: helical domain and Ras-like domain, also known as the G domain. Helical domain of G α proteins is shared and belongs to G α proteins, whereas the Ras-like domain is shared by all GTPases. G domain consists of 5 main sub-domains; G1, G2, G3, G4, and G5 (Figure 1.3). While the G4 domain

gives $G\alpha$ a specificity to bind to GTP/GDP, G1 and G3 domains have a function to bind to phosphates belong to GTP/GDP nucleotide. G2 is acting as an effector loop and coordinating Mg^{+2} ions. G5 domain act as a helper by strengthening GTP/GDP binding with its recognition sites (Cell Signalling: 3.5 Monomeric G Proteins - OpenLearn - Open University - S377_4, n.d.). Ras-like domain functions as GTP binding domain that binds to $G\alpha$ subunit after receptor activation and signal transduction. The helical domain of $G\alpha$ proteins specifies the $G\alpha$ binding proteins. After activation of $G\alpha$, the helical domain changes its conformation and loops displaced by Ras-like domain (Dohlman & Jones, 2012).

$G_{\alpha i}$ signaling is through inhibiting adenylyl cyclase by extracellular activation and conformational change of its specific GPCRs, thus cAMP cannot be produced from ATP. Moreover, this inhibition causes activation of PKA. cAMP pathways regulate processes in the body like heart rate, cortisol secretion, and breakdown of glycogen and fat. It is required to maintain memory, heart relaxation, water absorption in the kidney. Mainly, $G_{\alpha i}$ couples with acetylcholine M2 and M1, Adenosine A1 & A3 receptors, Cannabinoid receptors, Dopamine D2, D3, D4, serotonin and opioid receptors. $G_{\alpha i}$ signaling-related activation of Phospholipase C(PLC) and inhibition of adenylyl cyclase may cause an increase in vascular tone, and the diameter of vessels. Smooth muscle relaxation is also related to $G_{\alpha i}$ signaling. Signal transductions from endothelial cells via $G_{\alpha i}$ protein cause Nitric Oxide release and prostaglandins and endothelium-derived hyperpolarizing factors (EDHFs). This action causes relaxation in smooth muscle cells. Via adrenergic receptor Beta, G_i signal transduction decreases PKA activity resulting in calcium inhibition, which reduces contractile force (Hendriks-Balk et al., 2008) .

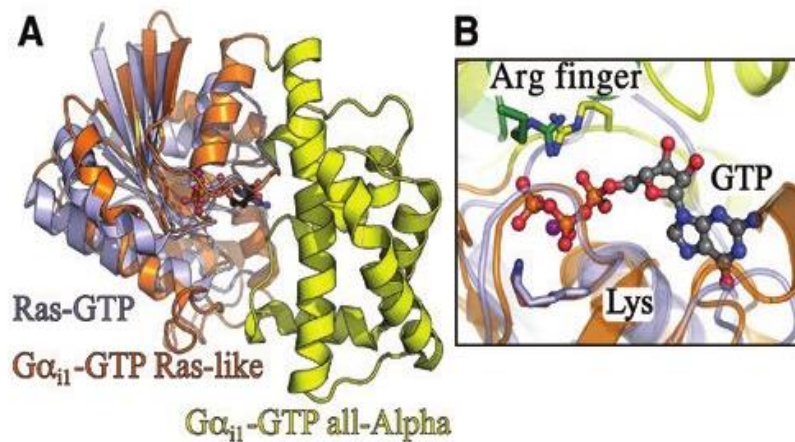


Figure 1. 4: Gai protein structure, A) overall 3D structure and domains and , B) GTP binding domain representation.(*Structural Similarities between Ras and Ga 11. (A) The Ga Subunit of... | Download Scientific Diagram, n.d.*)

In contrast, the Gas mechanism stimulates cAMP production when its GPCR is activated. ATP to cAMP production increases, so PKA gets activated. Related GPCRs to Gas signaling are mainly; ACTH receptors, Adenosine receptor types A2a and A2b, Arginine vasopressin receptor 2, β -adrenergic receptors types $\beta 1$, $\beta 2$ and $\beta 3$, Cannabinoid receptor 2, Dopamine receptors D1-like family. Gas subunit related signaling via beta adrenergic receptors mediate contraction in cardiomyocytes via PKA phosphorylation and Ca^{+2} channel (Hendriks-Balk et al., 2008). Activity increases Ca^{+2} leading to increase of positive charge intracellularly(London et al., 2004).

Gaq family members are Gaq, Gα11, Gα14 and Gα16. While Gaq and Gα11 show ubiquitous expression patterns and having 90 % sequence identity with each other, Gα14 express from kidney, liver, and lung cells and has 80 % sequence identity with Gaq. In addition, Gα16 express from hematopoietic cells and has 57 % identity with

Gαq(Mizuno & Itoh, 2009). Gα12 family consists of Gα12 and Gα13 and express by most human cells(Kelly et al., 2006) .

1.2 GPCR signaling

G protein-coupled receptors (GPCRs), which are integral membrane proteins, are the largest family of signaling receptors. 1/3 of all produced clinical drugs on the market target GPCRs (Hauser et al., 2017). While inactive GPCR signal transduction phase, GPCR and its heterotrimeric G protein locates from cell membrane. There are two main hypothesis for GPCR-G protein coupling. The first one claims that G protein-GPCR interaction occurs after ligand binding and then Gα and Gβγ subunit dissociates due to GDP-GTP exchange (*GPCR | Learn Science at Scitable*, n.d.). The second one claims that there is a GPCR-G protein precoupled complex. After ligand interaction, Gα and Gβγ subunit dissociation occurs due to GDP-GTP exchange.(Matúš & Prömel, 2018). Ligand interaction with GPCRs results in GPCR signalling activation and Gα dissociation from Gβγ. Last 11 amino acid of Gα subunit is required for heterotrimeric G protein GPCR interaction (Gilchrist et al., 2002). When the extracellular part of GPCR interacts with its ligand, change in its conformation triggers Gα subunit GDP-GTP exchange and heterotrimeric G protein is activated. Gα subunit separates from beta-gamma dimer, Gα subunit transduces signal via its effector interactions while beta-gamma dimer regulating other effectors (Nestler & Duman, 1999). This heterotrimeric G protein based secondary messenger system is required to transform extracellular signals to cellular responses (Morris & Malbon, 1999) (Tsvetanova et al., 2015).

1.2.1 Classical GPCR Interaction Model

The classical GPCR-G protein interaction model states one GPCR can interact with one heterotrimeric G protein. This model of GPCR signaling has seven steps. Firstly, GPCR, G protein, and adenylyl cyclase locate on the cell membrane with no interaction except random interactions. Then, agonist ligand of the GPCR bind and change GPCR conformation. Then, GDP-GTP exchange occurs at the alpha subunit of the heterotrimeric G protein. Activation of G protein causes $G\alpha$ and $G\beta\gamma$ separation. Separated $G\alpha$ protein interacts and activates adenylyl cyclase. Adenylyl cyclase binding site overlaps with $G\beta\gamma$ binding site. Thus, separation of $G\beta\gamma$ dimer from $G\alpha$ is required for the interaction with adenylyl cyclase. Activated adenylyl cyclase produces cyclic AMP. Finally, GTPase activity triggers GTP-GDP hydrolysis causing the termination of the signaling and heterotrimeric G protein reunites as presented in Figure 1.5.

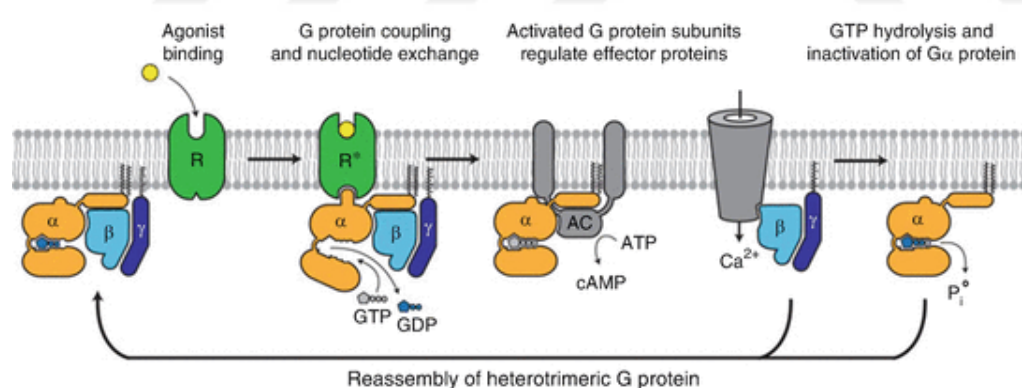


Figure 1.5: Schematic representation of Classical model of GPCR and heterotrimeric G protein interaction. (Taken from (Pincas et al., 2018))

1.2.2 GPCR oligomerization

The classical GPCR signaling model is still used, but recent studies have reported that GPCRs can oligomerize and share heterotrimeric G proteins while signaling (Ferré et al., 2016). This interaction type enables cell to control the signal intensity, enlarge transduced signal, modify the signal and cellular response as well. Oligomers could be homomeric with same kind of GPCR interaction or heteromeric more than one kind of GPCR interaction. Navarro and his group showed that A1 Receptor homodimer and A2A receptor homodimer interact with each other and form heterotetrameric structure. This heterotetramer allows G_{ai} and G_{as} protein interaction, theoretically. Furthermore, crosstalk between A2A homodimer and D2R homodimer was recognized in 1991, and interaction of these receptors was shown in 2015 (Ferré et al., 2016). According to this interaction model, it is not known whether G_{ai} and G_{as} are interacting or not.

1.3 G-protein signaling

G protein family, containing two subfamily as small G proteins and heterotrimeric G proteins, binds GTP and GDP molecules, act as a key role for signal transduction, cell growth and division, protein synthesis, and vesicular transportation (Wettschureck & Offermanns, 2005). G protein α subunit and small G proteins have structural similarities with each other and bind GTP and GDP similarly. In contrast with $G\alpha$ subunits, small G proteins can act independently as hydrolase enzymes (Sprang, 2016). While the monomeric structure is observed for small G proteins, heterotrimeric G proteins have subunits called $G\alpha$, $G\beta$ and $G\gamma$. When GPCR is activated by ligand and change its conformation, $G\alpha$ binds to GTP and then separates from $G\beta$ - $G\gamma$ heterodimer. While the $G\alpha$ subunit interacts with its effectors, $G\beta$ - $G\gamma$ dimer act on other effectors (Oldham & Hamm, 2008) As a result, activation of GTPases activates various pathways as presented in Figure 1.6 (Dohlman & Campbell, 2019).

Effectors of the heterotrimeric G proteins are mainly GIRK, MAPK, PLC β , PI3K, and adenylyl cyclase. In addition, small GTPase effectors are TIAM1, PI3K and RAF mainly. Both of these families has a role of cell growth, division and tumorigenesis since PI3K interaction. However, heterotrimeric G proteins regulates ion channels by interacting with GIRK and small GTPases regulates bipolar spindle assembly, chromosome congression and mitotic progression by interacting with TIAM1 (Monfort, 2018).

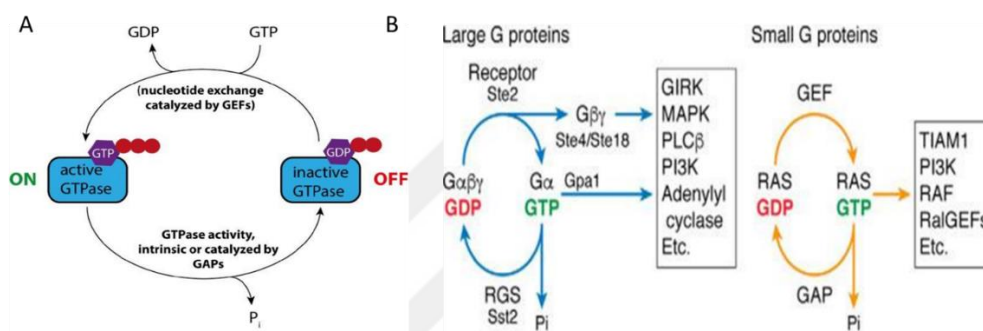


Figure 1. 6: Small G protein signal transduction. A) Schematic representation of Small GTPase signal transduction. B) Comparison of Small GTPase member Ras signal transduction with heterotrimeric G protein pathways. (Taken and adapted from (Monfort, 2018))

1.3.1 Signaling of Small GTPases

Small GTPases can act as monomeric hydrolase enzymes, bind their receptors and transduce signals. Ras proteins can be activated by Tyrosine kinase receptors, and GTP bound Ras protein transduces signal pathways like MAPK, PI3K that are important for cell differentiation and division as presented in Figure 1.7 (Vasan et al., 2014). Mutants of Ras proteins destroy the balance between differentiation and division. If cell division cannot be controlled, it could cause malignancy, and lead to cancer. K-Ras 4B mutants are seen widely on colorectal cancer. Recently, scientific

contributions about K-Ras 4B protein have been reported. K-Ras 4B mutant form can be seen with GNA12 protein wild type overexpression (H et al., 2015) and belongs to small GTPase family Ras group, binds each other, and creates dimers (Chung et al., 2016)(Nan et al., 2015).

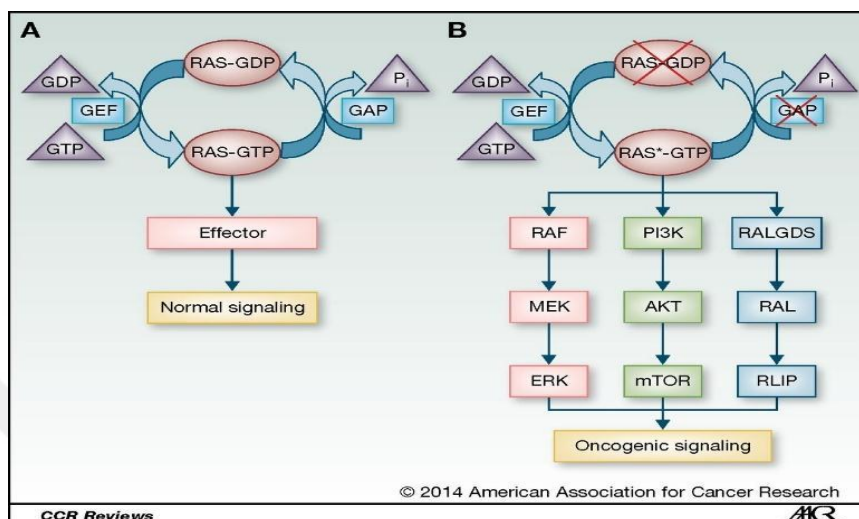


Figure 1. 7: K-Ras signalling pathways. A) Wild type Ras signalling pathways and, B) Oncogenic mutated Ras pathways (Taken from (Vasan et al., 2014))

1.3.2 Signaling of Heterotrimeric G protein

Heterotrimeric G protein α subunits also bind to their GPCRs, and GDP-GTP exchange system allows them to transduce signals. In addition, Navarro and his study group indicated that a heterotetramer structure of GPCR A1 homodimer and A2A homodimer interacts with each other and forms heterotetrameric GPCR oligomer (Navarro et al., 2016). In this heterotetrameric interaction, Gai protein is shared by A1 homodimer and Gas protein is shared by A2A homodimer. This system theoretically shows that, Gai and Gas proteins are close each other enough for a possible dimerization. A1 and A2A receptor heterotetramer model is schematically represented in Figure 1.8 (Franco et al., 2021)

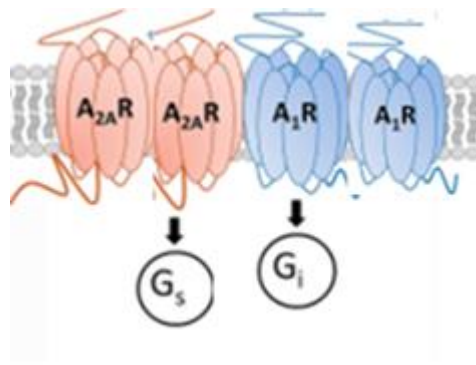


Figure 1. 8: A₁ and A_{2A} receptor heterotetrameric model coupling with G_{ai} and G_{as} proteins (Adapted from (Franco et al., 2021)).

1.4 Protein-Protein Interactions

Cellular pathways and responses from extracellular and intracellular events are mostly depend on proteins. In order to transduce signals that are responsible for such events via signaling pathways, proteins should interact with other molecules, mostly with other proteins. In order to say that two proteins are interacting, they should be closer than 10 nanometers (Xing et al., 2016a). These interactions between proteins are various, and proteins can interact with other proteins via random interactions. To be able to say that the two proteins are interacting specifically, there should be permanent or transiently contact to another molecule physically with a specific position and/or interface. Furthermore, in order to say that two proteins are interacting with each other specifically, interaction between two protein should have specific purpose as well , such as gene expression, protein degradation etc. (de Las Rivas & Fontanillo, 2010). According to their binding affinity, interaction can be obligate, which means one of the proteins from the complex is useless, so the complex form is required for the functionality. Whereas proteins can interact and form a complex, and also act independently in non-obligate interactions. According to interaction stability, interacting proteins can bind each other permanently or transiently, but most of the time, obligate interactions are permanent as given in Figure 1.9 (Acuner Ozbabacan et al., 2011).

Types of Protein-Protein Interactions

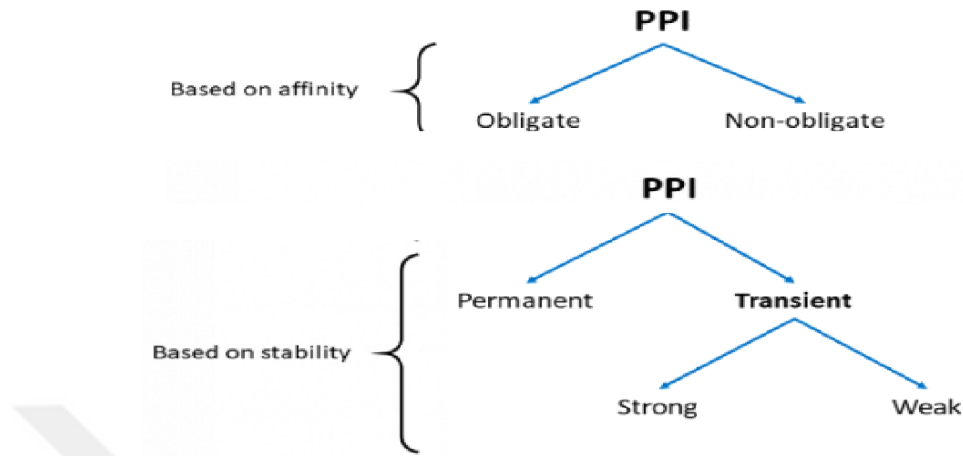


Figure 1. 9: Protein-protein interaction types. (Adapted from (Acuner Ozbabacan et al., 2011)).

1.4.1 Protein-protein Interaction detection Methods *in vivo*

Cellular events to sustain homeostases like synthesis, maturation, vesicle budding, trafficking, and degradation are controlled protein-protein interactions (PPIs) (Xing et al., 2016). Thus, tracking and detecting PPIs is an opened and essential field for systems biology. Especially in drug development and pharmaceutical fields, in order to detection of target molecules and their modification and affection , it is essential to know relevant protein-protein interaction outcomes *in vivo* (de Las Rivas & Fontanillo, 2010).

To detect PPIs *in vivo* there are two classes of methods that can be used. First class is high throughput techniques that include Yeast two-hybrid and affinity purification. The second class is low throughput techniques, that include devices as nuclear magnetic resonance (NMR) spectroscopy, atomic force microscope, and electron

microscope. Furthermore confocal fluorescent microscope could be used for FRET studies with proper fluorophore labeling while analyzing *in vivo* and *in vitro* protein-protein interactions (Peng et al., 2017).

1.4.1.1 Förster Resonance Energy Transfer (FRET)

Recent studies indicates that investigating interactions by using advanced fluorescent microscopy techniques is very popular and reliable (Ishikawa-Ankerhold et al., 2012). The advantage of such techniques is allowing the researcher to work *in vivo* and record and/or track live responses after certain treatments. FRET technique that belongs to advanced fluorescent microscopy techniques, is used widely to study for protein dynamics, protein-protein interaction and other biological molecules, such as DNA (Okamoto & Sako, 2017). Innovations in the fluorescence microscopy field, such as developing new fluorescent probes, allow scientists to use FRET technique more sensitively to be able to detect PPIs. Thus, FRET is a powerful tool to detect interactions and dynamics of proteins via its capability of detection sensitivity in nanosecond time range and angstrom distances with high resolutions in very broad and very narrow ranges.

FRET technique was developed in 1948 by Theodore Försters while he was aiming to study photosynthesis efficiency (Lemke & Deniz, 2011). FRET technique depends on nonradiative energy transfer between donor fluorophore and acceptor fluorophore. When donor fluorophore excited with its specific wavelength, it excites ground state non-excited acceptor fluorophore by long-range dipole-dipole interaction between donor and acceptor fluorophore that are located in between 10-100 angstrom distance (Sekar & Periasamy, 2003). In order to use FRET technique correctly, there are some requirements about donor and acceptor fluorophore selection. Firstly, the emission spectrum of donor fluorophore should have an overlapping region with the excitation spectrum of acceptor. Secondly, donor and

acceptor fluorophore distance should be more than the collision diameter. Lastly, dipole position between donor and acceptor should not be positioned.

Försters Theory suggests that energy nonradiative energy transfer rate $k_T(r)$ between donor and acceptor pair can be calculated by using the equation below:

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

From the equation, r means the distance between donor and acceptor, R_0 means Förster distance, and τ_D means the rate of decay of donor when acceptor fluorophore is absent. Thus, FRET efficiency calculation can be explained with the equation below:

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

Efficiency of the FRET formula enables to detect donor and acceptor distance, so its usage enables to calculate donor and acceptor distances in nanoscales (Wallace & Atzberger, 2017).

There are many ways to measure FRET Efficiency for example: Donor photobleaching (FLIM), Acceptor photobleaching, Spectral Imaging, and 3Cube method (Sensitized Emission). In this study, 3 cube method was used.

3 cube method is also referred as two-color ratio imaging (*Fluorescence Resonance Energy Transfer (FRET) Microscopy - Introductory Concepts* | Olympus LS, n.d.; Wallace & Atzberger, 2017). Overlaps between donor excitation-acceptor excitation and donor emission-acceptor emission are significant problems. The donor excitation with its specific wavelength should not excite the acceptor and the signal that is collected from acceptors emission spectrum should not overlap with donors emission spectrum ideally. Thus, 3 groups should be analyzed using proper filters to calculate FRET and eliminate the spectral bleed-through (SBT) from FRET data. First group is donor fluorophore expression only group, second group is acceptor fluorophore expression only group and the final one is FRET group with both donor and acceptor expression. Donor fluorophore expressed and excited groups emission signal that is collected from acceptor emission spectral region should be minimized and acceptor expressed groups donor excitation and acceptor emission signal should be minimized to collect ideal data. In this study, EGFP and mCherry fluorescent proteins are used as a FRET pair. Since overlaps between donor emission and acceptor emission and also donor excitation and acceptor excitation are very limited, by using certain regions for excitation and emission, crosstalk can be eliminated and ideal data can be collected (Figure 1.10).

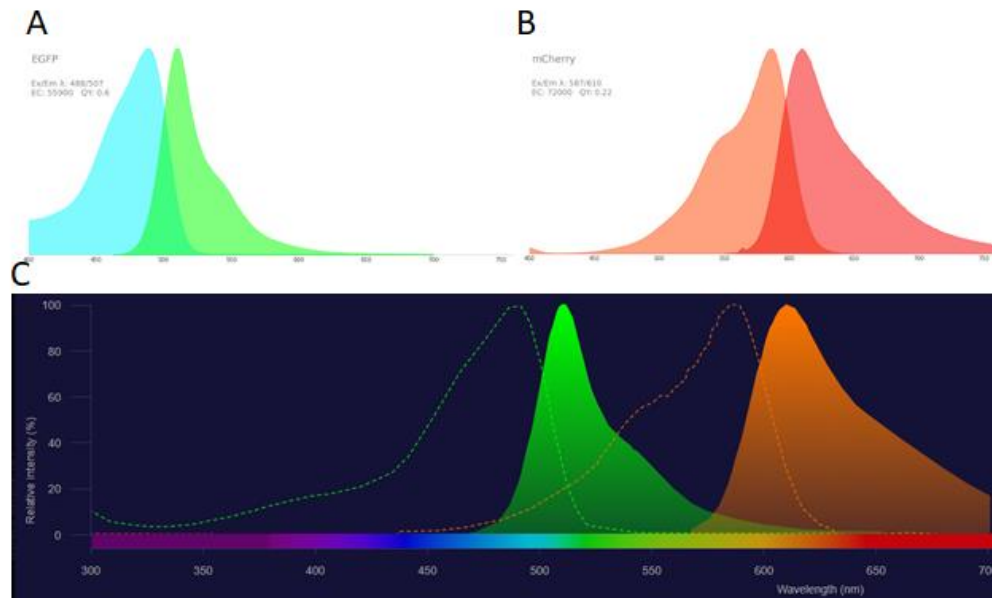


Figure 1. 10: EGFP and mCherry fluorophore excitation and emission spectrums. A) EGFP fluorophore excitation (blue) and emission (green) spectral graph, B) mCherry fluorescent protein excitation (orange) and emission(red) spectral graph, C) EGFP and mCherry spectral regions with overlaps. (Adapted from www.fpbase.org and <https://www.thermofisher.com/order/fluorescence-spectraviewer#!/>)

In this thesis, confocal fluorescent microscopy and monochromator plate reader were used for protein-protein interaction detection with FRET technique. Data taken from FRET experiments were calculated with FRET formula below.

$$FRET = I_{FRET} - BT_{Donor} * I_{Donor} - BT_{Acceptor} * I_{Acceptor}$$

$$NFRET = \frac{I_{FRET} - BT_{Donor} * I_{Donor} - BT_{Acceptor} * I_{Acceptor}}{N} * 100$$

$$BT_{acceptor} = \frac{I_{FRET}}{I_{Acceptor}}$$

$$BT_{donor} = \frac{I_{FRET}}{I_{Donor}}$$

1.4.1.1.1 Confocal microscopy fluorescent imaging

Most commonly used fluorescent microscope design in life sciences is epifluorescence design. Confocal microscope is an electronic system that is combined with a widefield epi-fluorescence instrument. This combination has laser illuminators, an electronic and optical component included scanning head, a computer system in order to display and monitor image and appropriate software to tune signal, process and analyze the images (Figure 1.11). Due to low signal to noise ratio of confocal microscope, it is one of the best device options to apply FRET technique.

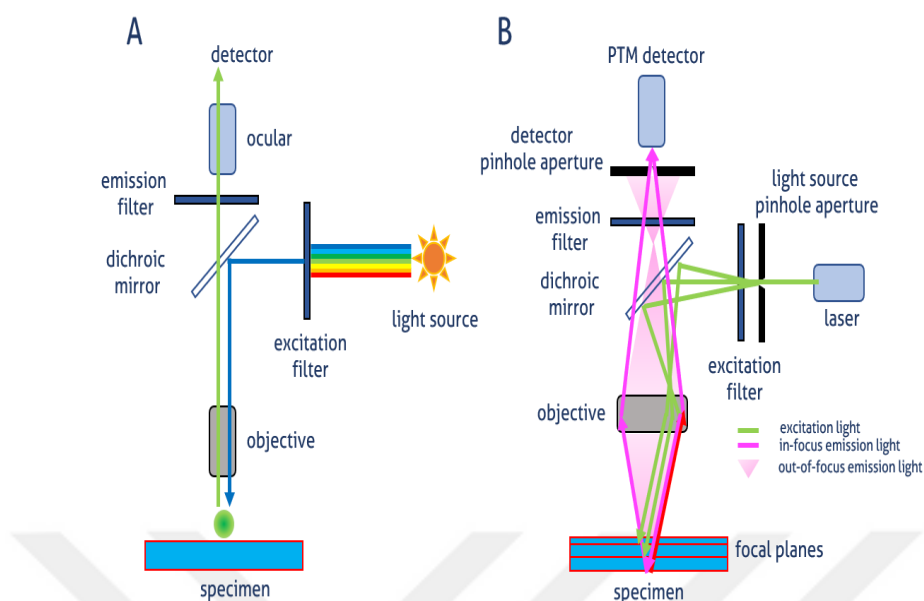


Figure 1. 11: Imaging principles of A)widefield epifluorescence and B)Confocal microscope (Taken from <https://www.ptglab.com/news/blog/if-imaging-widefield-versus-confocal-microscopy/>)

The sequential light collection is the main point of confocal imaging. Scanning of signal point by point by using Nipkow disc is one way to take an image with a confocal microscope. Nipkow disc enables the system to eliminate non-focused light by spinning and rotating the small holes. Thus, only focused light is expected to reach to the specimen, theoretically. As a result, both stage and beam stay stable, and also, the captured image is sharper and more detailed. In this thesis spinning disc confocal microscope was used for FRET imaging experiments (Figure 1.12).

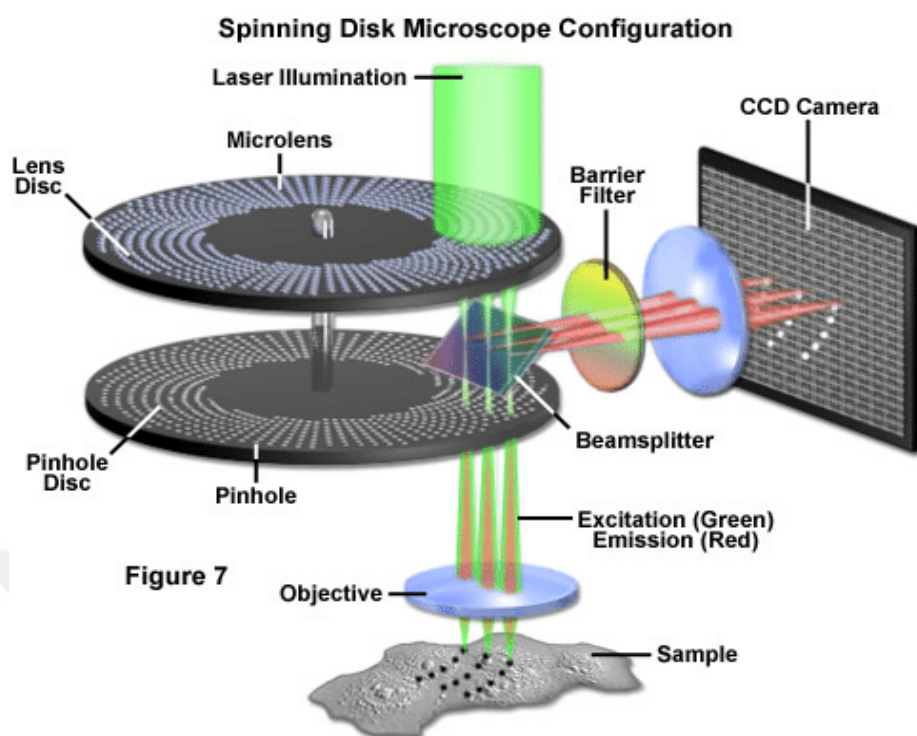


Figure 1. 12: Spinning disk microscope component schematic representation. (ZEISS Microscopy Online Campus | *Introduction to Spinning Disk Microscopy*, n.d.)

1.4.1.1.2 Monochromator Plate Reader

Monochromator plate reader diffracts light that is produced by the light source and enables the user to select specific wavelength excitation with the capacity of its slit width. The light for exciting the specimen passes from the excitation slit and reaches to the well containing sample. Later, emitted signal from the sample passes from the emission slit. In this thesis, using monochromator fluorescence micro plate reader for FRET applications enabled us to record FRET emission spectra and FRET spectral changes. In order to compare acceptor peaks and collect signals from FRET pair emission spectral regions, a negative control group was used.

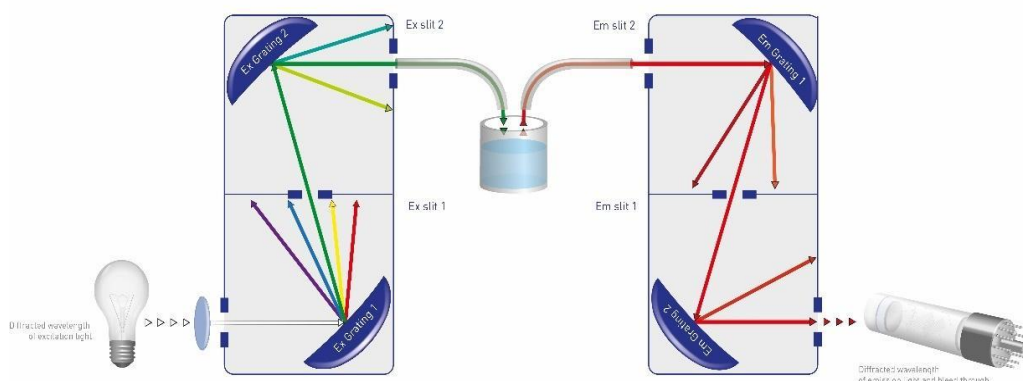


Figure 1. 13: Monochromator fluorescence microplate reader component schematic representation. (*Monochromator vs Filter-Based Plate Reader: Which Is Better?* - Promega Connections, n.d.)

1.4.1.2 Minigenes

G proteins interact with their GPCRs in order to transmit signals and initiate proper cellular responses. This interaction occurs via the last 11 amino acids of $G\alpha$ subunit. Many approaches have been developed in order to intentionally prevent this signal transduction for analysing the important components of the pathways. One of those approaches is minigenes. Minigenes used in this study are small cassettes that include ribosome binding sequence, stabilizing glycine amino acids and last 11 amino acid of relevant $G\alpha$ protein (Gilchrist et al., 2002). The function of ribosome binding sequence in this cassette is for the recruitment of the mRNA transcript of the minigene to a ribosome for minigene synthesis. Glycine amino acids at the beginning and the end in this cassette stabilize the peptide (Figure 1.14) (Gilchrist et al., 2002).

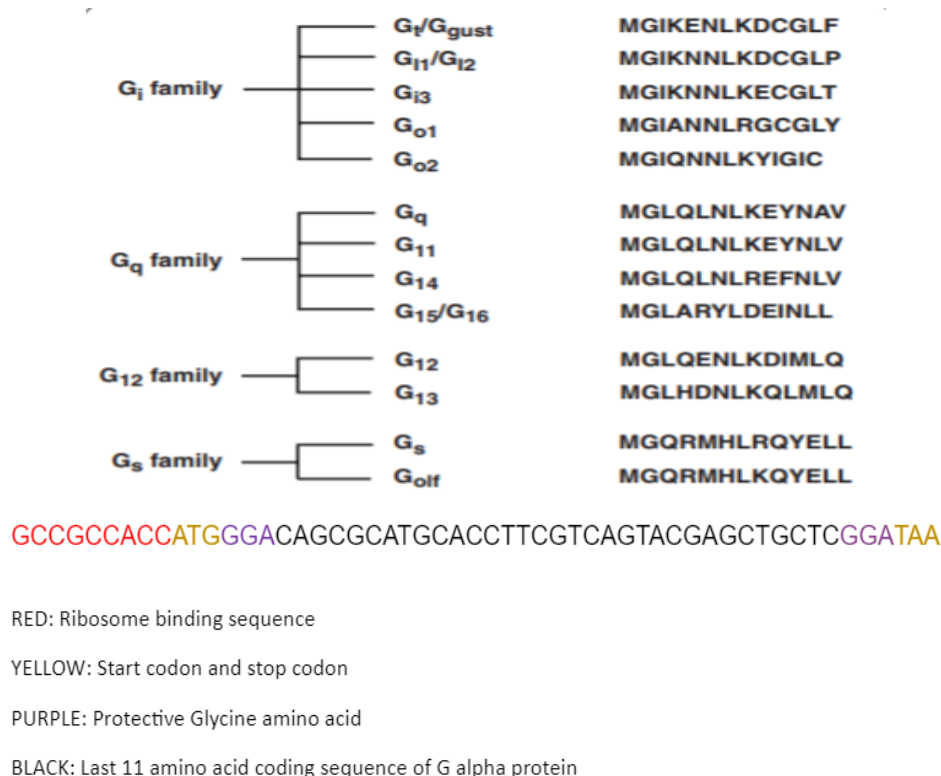


Figure 1. 14: G protein α subunit specific minigenes and sequence domains (Adapted from (Gilchrist et al., 2002)).

In this thesis, minigenes were used in order to inhibit the interaction between GPCRs and fluorescent protein labeled G α proteins. This approach enabled the investigation of receptor dependency on G α_i and G α_s protein interaction.

1.5 Aim of Study

Classical GPCR signaling suggests that, one GPCR can interact with one heterotrimeric G protein. However, recent studies reveal that GPCRs can form dimers and oligomers. GPCR oligomerization allows the cell to tune signaling and give an ability to the cell to respond more precisely. Many GPCR oligomer models were proposed by recent publications such as; A1-A2A heteromer and A2A-D2R

heteromer. In these oligomers, while A2A receptor has binding affinity to G α s protein, both A1 and D2R receptors has binding affinity to G α i protein.

Recent studies show K-Ras protein that belongs to small GTPase family activated by homodimerization (Muratcioglu et al., 2015; Nussinov et al., 2019). Muratcioglu *et al.* also studied this interaction in more details and represented two predicted interfaces for the dimer (Muratcioglu et al., 2015, 2020). In addition, Navarro *et al.* showed A1 and A2A GPCR heterotetramer model with theoretical G α s and G α i protein interaction. This interaction was shown by BRET method in their 2018 study (Navarro et al., 2018).

This study aims to analyze G α i and G α s protein interaction by using FRET technique. Effect of G protein - GPCR interaction on G α i-G α s interaction by the use G α protein specific minigenes and effect of ligand activation via A2A-D2R heterotetramer on G α i-G α s interaction was tested.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Mammalian Cell Culture

2.1.1.1 Mouse Neuroblastoma Neuro2a (N2a) Cell Line

In this study, to visualize and investigate the physical interaction between fluorescently tagged G α i and G α s proteins, the N2a cell line was used. N2a cell line cultivation and proliferation processes can be maintained and completed in 2-3 days. Furthermore, transfection applications can easily be performed by using the N2a cell line. In this thesis, an N2a cell line product called CCL-13 *Mus musculus* brain neuroblastoma was purchased from the ATCC company, ENGLAND.

2.1.1.2 Cell Media

N2a growth media was prepared by using OptiMEM®I Reduced Serum Medium with L-glutamine (Invitrogen, Cat#31985047) that makes 44.5% of the media, Dulbecco's Modified Eagle Medium DMEM including high glucose with L-glutamine (Invitrogen, Cat#41966029) that makes 44.5% of the media, 10% of the media is Fetal Bovine Serum (Biological industries, Cat#04127-1B) and 1% of the media is Penicillin/Streptomycin solution (Biological industries, Cat# 03-031-1B). The prepared N2a media was filtered using a 0.22 μ m filter. The formulation of DMEM is given in Appendix A.

2.1.1.3 Maintenance

Maintenance of the N2a cell culture required an incubator that can be used at 5 % CO₂ and 37 °C. In addition, passage and transfection processes were performed by using Nuve® LN 120 laminar flow cabinet. The passage of the cells was performed every 3 days after reaching 80 % confluency. During the passage, before removing cells from the attached surface, the washing step was performed by using PBS. The passage of N2a cells required Trypl-E Express with phenol red (Invitrogen, Cat#12605-028) and the passage of the cells was performed in the T25 flask. Formulation of PBS was given in Appendix A.

2.1.1.4 Other Chemicals and Materials

Transfection protocol performed by using Lipofectamine™ LTX and Plus™ Reagent purchased from Invitrogen (MA, USA), using the optimized protocol below.

2.1.2 Bacteria culture

2.1.2.1 Strain of Bacteria and Bacterial Growth Media

Bacterial transformation experiments were performed by using *Escherichia coli* XL1-Blue strain. As bacterial growth culture media, Luria Bertani solution was prepared and autoclave sterilized for 20 minutes by using 121 °C from Nuve® OT 40L autoclave machine. In order to apply bacterial selection with antibiotics, ampicillin (100 µg/ml) was used. Transformation of bacterial colonies was collected from LB agar plates incubated after transformation protocol at 37 °C for 16 hours from the ZHWY-200B incubation machine by Zhicheng Instruments. The incubation process of the collected colony inoculation was performed by using liquid LB

solution and incubated into rotary shaker ZHWY-200B by Zhicheng Instruments at 200 rpm for 16h at 37 °C.

2.1.3 Cloning

2.1.3.1 Plasmids, Primers, and Sequencing

Plasmids containing *Homo sapiens* Gai1 and Gas protein genes and also EGFP and mCherry labeled versions prepared within the scope of TÜBİTAK project numbered 117Z868 and titled as “Investigation of Gα protein dimerization mechanisms in live cells”. Unlabeled (carrying wild-type genes) versions of these plasmids were purchased from Addgene. FRET control groups were prepared in our laboratory by a former Master student Hüseyin Evci. EGFP and mCherry plasmids were kindly gifted by Prof. Dr. Henry Lester, California Institute of Technology (CA, USA). The mammalian expression vector containing CMV promoter used in this thesis is pcDNA3.1(-) that was kindly gifted by Prof. Dr. Ayşe Elif Erson Bensen, Middle East Technical University (Ankara /TURKEY).

Primers used in this study were purchased from Integrated DNA Technologies (IDT) (IO, USA) and PRZ Biotech (Ankara, TURKEY). All constructs were sequenced by BMLabosis (Ankara/TURKEY).

2.1.3.2 Other Chemicals and Materials

Chemical materials were purchased from Sigma Chemical Company (NY, USA). Molecular cloning enzymes like Phire Green Master Mix DNA polymerase, T4 ligase, and 6X DNA dye purchased from Thermo Scientific (MA, USA) and restriction enzymes purchased from New England Biolabs (MA, USA). Generuler

was purchased from Fermentas. Plasmid Miniprep and Gel extraction kits were purchased from Thermo Scientific (MA, USA).

2.2 Methods

2.2.1 Cloning

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

In order to prepare competent *E. coli* XL1-Blue cells, firstly, cells were streaked on an antibiotic-free LB agar plate and incubated for 16 hours at 37 °C. After the incubation period, a single colony was picked and inoculated with 2 ml antibiotic-free liquid LB media. Inoculated growth tube was incubated from shaker for 16 hours at 37 °C, 200 rpm. Then the overnight grown culture was inoculated into antibiotic-free LB culture media containing 20 mM MgSO₄ with a 1:100 (v:v) ratio. Prepared inoculation flask incubated into shaker at 37 °C, 200 rpm until reaching 0.4-0.6 OD 600. When the subculture reach to OD 0.4-0.6 at 600 nm, the culture was separated into 50 ml falcons and centrifugated at 4000 rpm at 4 °C for 5 minutes. After the centrifugation process, the supernatant was discarded, and the pellet was gently dissolved in 20 ml TFB1 solution then incubated for 5 minutes on ice. After that, the pellet dissolved in TFB1 solution and centrifued at 2000 rpm for 5 minutes at 4 °C. The supernatant was discarded and pellet gently dissolved by using 2 ml TFB2 solution. The falcons incubated on ice for 45 minutes. After the incubation, cells were aliquoted as 50 µl into 1.5 ml Eppendorf tubes and quickly transferred into liquid nitrogen. The competent cell containing Eppendorf tubes were then stored at -80 °C. TFB1 and TFB2 solution preparation was explained in Appendix A.

2.2.1.2 Polymerase Chain Reaction (PCR)

In this study, the PCR method was used for EGFP and mCherry fluorescent protein amplifications for Gα tagging and minigene sequence amplification.

For Gα tagging, fluorescent protein amplicants include 24bp overlapping regions with Gai and Gas proteins from both 5' and 3' regions. Between overlapping region and EGFP/mCherry sequence, there are 18bp linker sequences (TCTGGAGGAGGAGGATCT). Primer designs were given in Appendix C. Optimal PCR condition was given in Table 1.

For Gα specific minigene construction, gene cassette with the last 11 amino acid sequence of Gα proteins amplified by using PCR mixture given in Table 2.4 and conditions in Table 2.2 and 2.3. Since G-C content was very high, no annealing step was used in PCR for Gas minigene.

Table 2. 1:Optimal PCR conditions for Gα tagging

Reagent	Amount			
5X Phire Reaction Buffer	10μl	Pre-denaturation	98 °C	30 sec
Phire HS II DNA Polymerase	1 μl	Denaturation	98 °C	10 sec
DNA Template(EGFP/mCherry)	250-300ng	Annealing	58 °C	30 sec
10Mm dNTPs	1 μl	Extension	72 °C	1 min
Forward Primer	1.25 μl(20pmol)	Final Extension	72 °C	5 min
Reverse Primer	1.25 μl(20pmol)			
DMSO	1.5 μl(3%)			

X 34

Nuclease Free Water	Up to 50 μ l
Total	50 μ l

Table 2. 2: Optimal PCR conditions for Gai Minigene Preparation

Pre-Denaturation	98°C	30 sec.
Denaturation	98°C	10sec.
Annealing	55°C	30 sec.
Extension	72°C	10sec.
Final Extension	72°C	1 min.

Table 2. 3: Optimal PCR conditions for Gas Minigene Preparation

Pre-Denaturation	98°C	30 sec.
Denaturation	98°C	10sec.
Extension	72°C	10sec.
Final Extension	72°C	1 min.

Table 2. 4: PCR mixture for Gα minigene Preparation

Reagent	Amount
Phire Green Master Mix	10 μ l
Gai / Gas wt in pcDNA3.1 (-)	100ng
Forward Primer	0.25 μ l from 20 μ M stock
Reverse Primer	0.25 μ l from 20 μ M stock
Nuclease free water	Up to 20 μ l

2.2.1.3 PCR Integration Method (Overlap Extension PCR Method)

Fluorescent protein amplicants with overhanging regions that match with G protein α subunits were inserted into G α proteins. Confirmed amino acid positions of the insertions are A121-E122 and L91-K92 for G α_i and E73-S85 for G α_s proteins. For this insertion method, fluorescent proteins with proper overhang regions that match the appropriate G α gene should produce and amplified by using PCR. For the second PCR reaction, those amplicants were used as megaprimers with a 1:5 template primer ratio. Matched regions of the mega primer bind the G α gene matching region and produce a whole new artificially produced non-methylated plasmid. All methylated unlabeled template plasmids were digested via DpnI. The conditions of the method and a schematic figure about the method were given in Table 2.5 and Figure 2.1.

Table 2. 5: Optimized integration PCR protocol

Reagent	Amount			
Phire Green HS II Master Mix	10 μ l	Pre-denaturation	98 °C	3 min
DNA Template(G α_i /s in pcDNA 3.1(-))	100ng	Denaturation	98 °C	30 sec
1st PCR products	500ng	Annealing	51-65 °C	1min
DMSO	1.5 μ l(3%)	Extension	72 °C	2 min/kb
Nuclease Free Water	Up to 50 μ l	Final Extension	72 °C	5 min
Total	50 μ l			

X 18

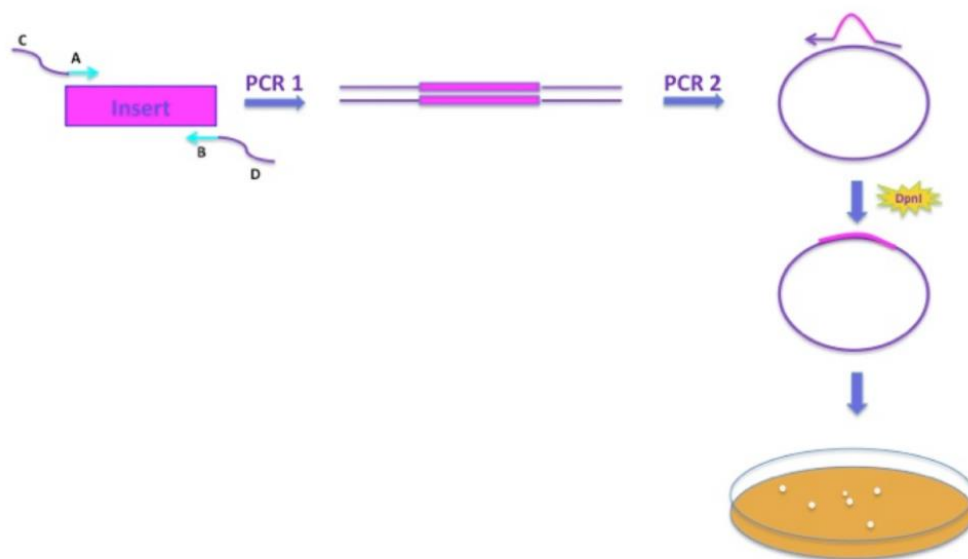


Figure 2. 1: Overlapping extension PCR representation. A and B parts of PCR primer representation fit with the insertion sequence, while C and D parts fit the vector that insertion was performed. (Taken from <https://bitesizebio.com/20958/overlap-extension-pcr-cloning/>)

2.2.1.4 Agarose Gel Electrophoresis

DNA samples amplified via PCR and restriction products for fluorescent protein tagging were visualized to control and validate amplification by using 1 % agarose gel. On the other hand, samples of DNA for minigene preparation were visualized via 2 % agarose gel. The agarose gels were prepared as 100 ml volume and 36 μ l Ethidium Bromide (EtBr) added into the gel as DNA chelator to visualize using UV light. Firstly, 1 gram of agarose for 1 % or 2 grams of agarose for 2 % gel was weighted and added into 100 ml 1X TAE buffer. The mixture was heated with microwave until the agarose pieces were dissolved. Then liquid agarose gel mixture was cooled with tap water for 30 seconds, and after cooling, 36 μ l EtBr was added and mixed well. The gel was poured into a gel box with appropriate combs. After polymerization, DNA samples were prepared via diluting loading dye (Thermo

Scientific, #R0611) from 6X to 1X. The samples were loaded into wells, and Generuler (Fisher, Product code:11803983) was used as a DNA marker. The Samples run for 35 minutes with 110V. 50X TAE buffer stock solution preparation protocol can be found in Appendix A.

2.2.1.5 Extraction from Agarose Gel

Amplification of PCR products and restriction products were loaded to agarose gel and visualized via UV. Comparing with the appropriate position of the DNA ladder, DNA bands were cut and placed into 1.5 ml Eppendorf tubes. The gel contained Eppendorf tube weight was measured, and empty Eppendorf tube weight was subtracted from it. GeneJET Gel Extraction Kit (Thermo Fisher Scientific, #K0691) was used to extract DNA from agarose gel, and the protocol given by the kit was applied. In the final step, 30 μ l preheated nuclease-free water was used for DNA elution from the column membrane instead of the 50 μ l Elution Buffer provided by the kit to increase eluted DNA yield.

2.2.1.6 Determination of DNA Amount

In order to detect the yield of DNA products, BioDrop μ LITE device was used. Before measurement, the sample loading space was washed via distilled water to clean the remaining samples from other measurements. Then device opened and calibrated itself. Life sciences section and DNA subsection were selected and 1 μ l nuclease-free water was used as blank. After blank measurement, 1 μ l DNA sample was loaded on sample loading space, and the measurement was taken.

2.2.1.7 Restriction Enzyme Digestion

New England Biolabs Inc. (NEB) (MA,USA) restriction digestion enzymes were purchased for this study. Sticky ended products were obtained after the restriction

process. As NEB's instruction manual suggested, the restriction process was performed with 1 unit of enzyme for 1 µg of DNA. In this study, at least 1000 ng DNA was used for the restriction processes with 1 µl enzyme and 1.5 µl CutSmart® NEB buffer, and the final volume was completed to 20 µl. The final mixture mixed well and incubated for 4 hours at 37 °C.

2.2.1.8 PCR Purification

In order to remove restriction components and purify the DNA product, PCR purification was applied by using the Thermo Scientific GeneJet Purification kit (#K0702). The protocol given by the kit was applied, but the elution step was performed by using 30 µl nuclease-free water instead of 50 µl EB to increase yield.

2.2.1.9 Ligation

Ligation reaction was used to covalently bind the DNA products and the vector plasmid that are restricted compatibly. In this study, the ligation process was applied to insert sticky-ended restriction products into expression vector pcDNA3.1(-). Vector to insert ligation ratio as 1:3, 1:5, 1:10, and 1:15 was calculated according to the 150 ng restricted vector. 20 µl ligation reaction includes at least 150 ng restricted vector, insert, 1µl T4 DNA Ligase enzyme (NEB, Cat#0202T) and 1X 2 µl T4 DNA ligase buffer, and then the total volume was completed to 20 µl by using nuclease-free water. The ligation reaction was incubated at room temperature for 16 hours.

2.2.1.10 Transformation of Competent *E. coli* Cells

Bacterial transformation protocol applied for transferring a vector that carries desired gene into a bacterial host. In this study, this protocol was applied to ligation products and plasmids that need to be amplified. RbCl competent *E. coli* XL1 blue strain used

as a bacterial host that was stored at -80 °C. Competent cells were taken from -80 °C and kept on ice for 15 minutes. After 15 minutes of incubation time, 7 µl ligation product and 1 µl circular plasmid were added into a competent cell tube, pipetted well under sterile conditions. The mixture of DNA and bacteria was incubated on ice for 30 minutes. Later, the tube containing DNA and competent cells were heat-shocked at 42 °C for 45 seconds. Then the tube was incubated on ice for 3 minutes. After incubation, 750 µl SOC was added into the tube, and the tube was incubated for 1 hour on shaker at 37 °C, 180 rpm. After the tube was centrifuged at 4000 rpm, 600 µl supernatant was discarded and the pellet was dissolved with the remaining SOC. The dissolved pellet was spread on LB agar plate prepared with ampicillin since the vector used in this study (pcDNA3.1(-)) has an ampicillin resistance gene. Agar plates were incubated at 37 °C for 14-16 hours for bacterial colony growth.

2.2.1.11 Plasmid Isolation from *E. coli*

Plasmid DNA isolation protocol was performed by using Thermo Scientific's GeneJET plasmid miniprep kit and protocol provided by the kit(#K0503). Single colony was selected on the agar plate and inoculated into 5ml LB with 5 µl ampicillin and worked sterile. LB with antibiotic and bacteria colony was incubated at 37 °C for 16 hours with 180 rpm. Later, the LB tube centrifuged at 4000 rpm. Supernatant discarded and protocol provided by the kit was applied on the pellet. The elution step was performed by using 70 µl nuclease-free water instead of 100 µl EB to increase yield.

2.2.2 Mammalian Cell culture

2.2.2.1 Passage And Cell Seeding

In this study, Neuro2A (N2A) cell line from *Mus musculus* was used as a mammalian cell line. N2A cells have approximately 70 hours of doubling time, so passage protocol was applied after 3-4 days of incubation. During the incubation time, N2A cells were grown into a T25 flask and incubated at 37 °C and 5% CO₂. For passaging process, the flasks were taken into the laminar flow, the cell media in flask was suctioned, and cells were washed gently by using warm 1X PBS. Later, PBS was suctioned and 500 µl Trypl-E was added to the top of the cells. The flask was transferred into 37 °C incubator with 5% CO₂ for 5 minutes. Then 8ml N2A media was added into the incubated flask in the laminar flow, pipetted gently. New T25 flask labeled and 8ml N2A media was added into it. 400 µl cell media with N2A cells were taken from the old flask and added into the prepared flask. The new flask was incubated at 37 °C incubator with 5% CO₂ until the next passage. These cells were used until the 45th passage.

The cells from the old flask were kept, and 10 µl of the cell suspension was transferred onto hemocytometer with a coverslip. Under microscope (100X), the cells were counted from the 4 corners, and then total number was divided to 4 and multiplied to 10.000 to find the cell number in 1ml suspension. 120.000 cells for 35mm plastic dishes and 60.000 cells for 35mm glass bottom dishes were calculated and seeded. Glass bottom dishes were used for microscope imaging and plastic bottom dishes were used for plate reader experiments.

For plate reader experiments, after 1 day of transfection incubation, cells were lifted and 10.000 cells were seeded into 96 well plates.

2.2.2.2 Transfection of mammalian expression vector to N2a cells

In order to artificially introduce DNA samples into mammalian cells, a transfection protocol was used. Transient transfection performed by using Lipofectamine™ LTX with Plus™ Reagent purchased from Invitrogen (MA, USA) for this thesis study

since it this product has more than 80 % transfection efficiency rate (Maurisse et al., 2010). For the transfection process, 60.000 and 120.000 cells were seeded on glass and plastic dishes, respectively, followed by 2 ml media addition. The dishes were incubated for 24 hours before transfection process for cell attachment. Following day, 100-300 ng of plasmid DNA for microscope imaging and 100-500 ng of plasmid DNA for plate reader experiments was added into 100 μ l OptiMEM™ and mixed. 4 μ l Plus™ reagent was added into a plasmid and 100 μ l OptiMEM™ containing tubes. The mixture was incubated for 15 minutes at room temperature. During the incubation time, a new set of 100 μ l OptiMEM™ tubes were prepared. 4 μ l Lipofectamine™ LTX was added into tubes that were contained only OptiMEM™. After the incubation time, the tubes containing lipofectamine and added 100 μ l OptiMEM™ were mixed with the tubes containing Plus™ Reagent contained and 100 μ l OptiMEM™ with plasmid DNA. The final mixture was incubated for 15 minutes at room temperature. During incubation, dishes with cells were taken from the incubator, and the media was suctioned from the dishes. The cells were gently washed with 1ml 1X PBS. 1ml PBS was suctioned, and 680 μ l OptiMEM™ was added on top of the cells. When the incubation time ended, 200 μ l transfection mixture was dropped into cells with 680 μ l OptiMEM™. The dishes were incubated at 37 °C with 5% CO₂ for 3 hours and 2ml media was added afterward. For confocal microscopy experiments, the dishes were incubated for 2 days for imaging and were used afterwards. For plate reader experiments, dishes were incubated 1 day and then cells were lifted and seeded into 96 well plate.

2.2.3 Fluorescence Measurements

2.2.3.1 Imaging with Spinning Disc Confocal Microscope

After transfection of EGFP and mCherry tagged G α proteins to N2A cells, and following incubation time, glass-bottom dishes were imaged by using Leica DMI 4000 equipped with Andor DSD2 spinning disc confocal microscope. The reason for

the usage of the Andor DSD2 spinning disc microscope is that it has a frame rate maximum of 22 frames per second with 370 – 700 nm excitation range and 410 – 750 nm emission range. The spinning disc enables the rejection of non-focused light and gives sharper images. The imaging process was performed by using 63X immersion oil NA 1.4 objective lens.

For confocal microscopy FRET experiments, 3-Cube method was used. Plasmids were transfected into 60.000 N2a cells on the 35mm glass bottom dishes. For each FRET pair, three dishes were prepared. The first dish contained only Gα protein gene tagged with EGFP sequence, the second one contained Gα protein gene tagged with mCherry sequence, and the third one contained both EGFP and mCherry tagged Gα protein genes. First and second dishes used for bleed through calculations.

For bleed through dishes, two imaging setups were used, and images were taken as stacks of both images. Only EGFP tagged Gα protein containing dishes were excited with 470-500 nm wavelengths, and emission spectrums were set as 500-550 nm that collects green signal (donor channel) and 600-650nm that collects red signal (FRET channel). Only mCherry tagged Gα protein containing dishes were excited with 560-600 nm (Acceptor Channel) and 470-500nm, and the emission spectrum was set as 600-650 nm that collects red signal. All three imaging setups were used for FRET dish, and images were taken as stacks containing three images.

2.2.3.2 Measurement with Monochromator plate reader

After imaging and visualization with a confocal fluorescent microscope, Fluorescence microplate reader measurements were taken. While imaging with a confocal fluorescent microscope, approximately 50-100 cell images were collected, but 96 well plate measurements can give a signal combination of 10.000 cells; therefore, the results would more accurately represent the whole population of transiently transfected cells. In addition, the fluorescence plate reader enables to

arrange specific excitation wavelengths according to its slit width. In this study, SpectraMax iD3 Multi-Mode Microplate Reader (Thermo Scientific) was used as a monochromator plate reader with 450 nm excitation for donor fluorophore EGFP, and 490-750nm spectral emission to collect overall EGFP and mCherry emission spectrum signals. For FRET measurements, only donor, only acceptor, and FRET groups were transfected to N2A cells and 10.000 cells seeded into SPL black 96 well plates. If there is an energy transfer between donor and acceptor fluorophores EGFP and mCherry, normalized spectrum gives lower EGFP peak and increased mCherry peak. Therefore, spectral emission signals were normalized and EGFP and mCherry peaks were compared to investigate if there is an energy transfer or not.

2.2.4 Image Analysis with Pix-FRET Program

The physical interaction of proteins can be investigated by using the FRET technique. Although many advantages of FRET over other methods like enabling live-cell investigations, spectral bleed-through should be in consideration. When donor and acceptor excitation and emission spectrum overlap, excitation of the donor may also excite the acceptor, and donor emission spectra may leak to acceptor fluorophore emission spectra. These situations cause false FRET signals and are called spectral bleed through. For FRET experiments, three groups were used; the first one expresses only donor fluorophore tagged protein, the second one expresses only the acceptor fluorophore tagged protein, and the last group is the FRET group that expresses both of these proteins. In this thesis study, the Pix FRET plug-in of ImageJ was used for FRET calculations of confocal microscope images (Ro^{mero} *et al.*, n.d.). PixFRET allows to eliminate false FRET signals by analyzing signals from the only donor expressing and only acceptor expressing cell images used (Ro^{mero} *et al.*, n.d.). During analysis to determine spectral bleed-through, only donor and only acceptor images were loaded into ImageJ program PixFRET plug-in. Ten FRET group images were analyzed, and FRET efficiencies were determined cell by cell (Ro^{mero} *et al.*, n.d.)

2.2.5 Functional Analysis with cAMP-Glo™ Assay

The functionality of fluorescent protein labeled Gα proteins produced in TÜBİTAK 1001 project 117Z868 and titled “Investigation of Gα protein dimerization mechanisms in live cells” analyzed with cAMP-Glo™ assay produced by Promega (WI, USA). The assay principle depends on cAMP concentration measurement in a cell. If cAMP concentration increases in cells, cAMP binds to protein kinase A(PKA). There are two catalytic and two regulatory domains of PKA. If there is no cAMP in the cellular environment, PKA remains in the inactive form. During activation and cAMP production, the regulatory component changes its conformation, and the catalytic component is released. The released catalytic subunit catalyzes ATP transfer terminal and produces PKA substrate. Therefore, unused ATP level measured with cAMP-Glo assay with Kinase Glo reagent, which is a luciferase-based reagent. Concentration changes of ATP can be determined as light intensity. In addition, the cAMP level is inversely proportional to the luminescence signal. When cAMP increase, the luminescence signal decrease (Figure 2.2).

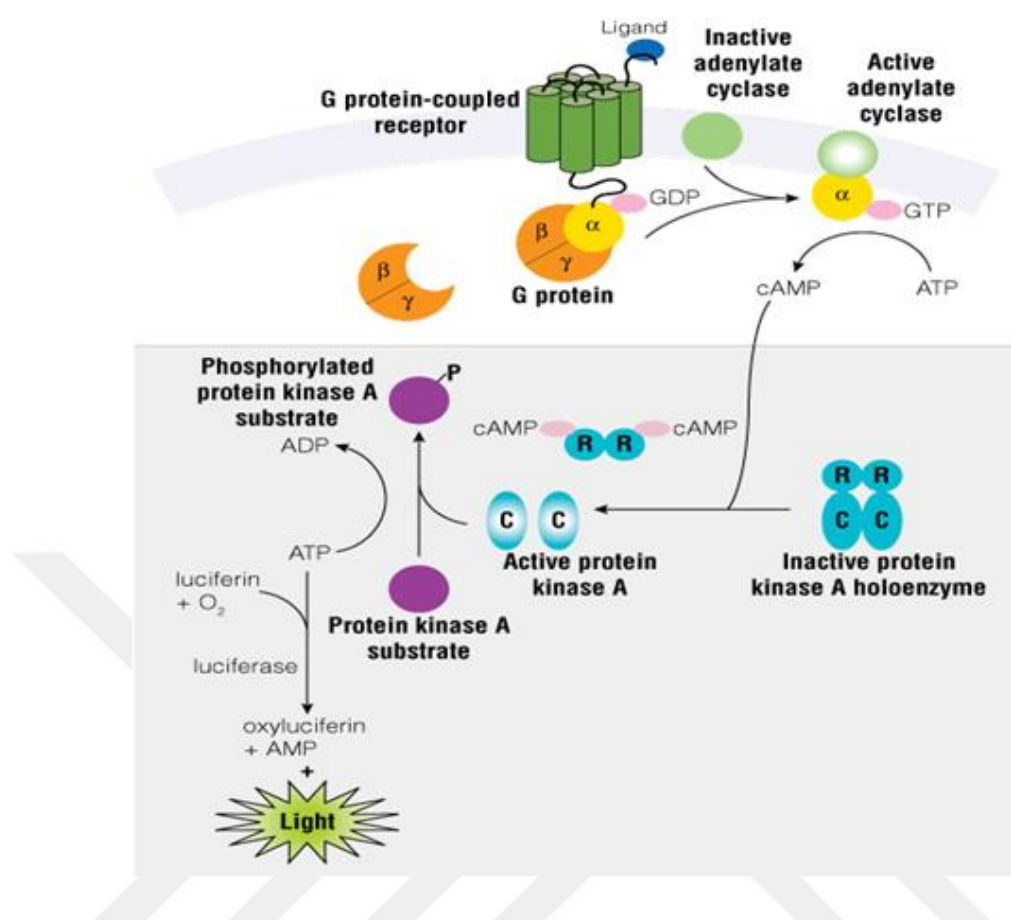


Figure 2. 2: cAMP-Glo™ Assay kit working principle. (Taken from www.promega.com)

Gai protein activation with D2R results in inhibition of cAMP level while Gas protein activation with A2A receptor increases cAMP concentration. Therefore, the activity of Gai protein causes an increase of luminescence signal while Gas protein activity results in a decrease.

In this study, 120.000 N2A cells were seeded onto 35 mm plastic bottom dishes and incubated for 24 hours. After incubation time, 500 ng plasmids of fluorescently labeled Gα proteins were transfected via Lipofectamine LTX with Plus Reagent, and incubated for 24 hours. Then, cells were lifted and counted. 10.000 cells were seeded on 96 well white SPL immunoplate. The next day, before kit protocol, Gαi transfected cell containing wells were treated with 20 µl of 20 µM forskolin (Sigma-Aldrich, #66575-29) for the cAMP increase and incubated for 30 minutes at 37 °C

with 5% CO₂. Then, forskolin was removed and 10 µM Quinpirole (Sigma-Aldrich, #73625-62) dissolved into 1X induction buffer (formulation in Appendix A) added into wells as D2R agonist, incubated at 37 °C with 5% CO₂ for 20 minutes (DM et al., 2014). For Gαs expressing cells, 15.5 µM 20µl CGS 21680 (Sigma Aldrich, product# 119137) dissolved into 1X induction buffer added as A2A receptor agonist and incubated for 20 minutes at 37 °C with 5 % CO₂ (D et al., 2011). After the A2A receptor and D2R activation with proper agonists, assay protocol was performed according to the manual given by the supplier.

2.2.6 Statistical analysis

Graphpad Prism is computer software used by scientists for basic statistical analysis of scientific data. The program enables its users to combine graphs, calculate statistical analysis, and organize data. In this thesis study, data analyses were performed by using Graphpad prism 8. To analyze the data with Graphpad Prism 8, One Way ANOVA was used to analyze more than two groups with one condition. T-Test used to compare two different groups with one condition. Results were presented as mean ± standard error of the mean (SEM). P values less than or equal to 0.05 are considered significantly different.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Functional Analysis of EGFP and mCherry Tagged $G\alpha$ proteins by cAMP-Glo Assay Kit

In this thesis, $G\alpha i$ proteins that were labeled between 91st-92nd and 121st-122nd positions and $G\alpha s$ protein that was labeled from 73-85 position with mCherry and EGFP were used as FRET pairs. These constructs were prepared by a former Lab. member Ms. Özge Atay. The functionality of $G\alpha s$ protein that was labeled with EGFP and mCherry proteins, and $G i$ 121 EGFP were tested with cAMP -Glo Assay Kit purchased from Promega (WI, USA) in our laboratory during previous studies. $G\alpha i$ 91-92 EGFP and mCherry and $G\alpha i$ 121 mCherry functionalities were tested with the same kit during this thesis period. The results obtained from these functionality analysis presented in Figure 3.1.

Functional analysis of Fluorescently Labeled $G\alpha$ Proteins

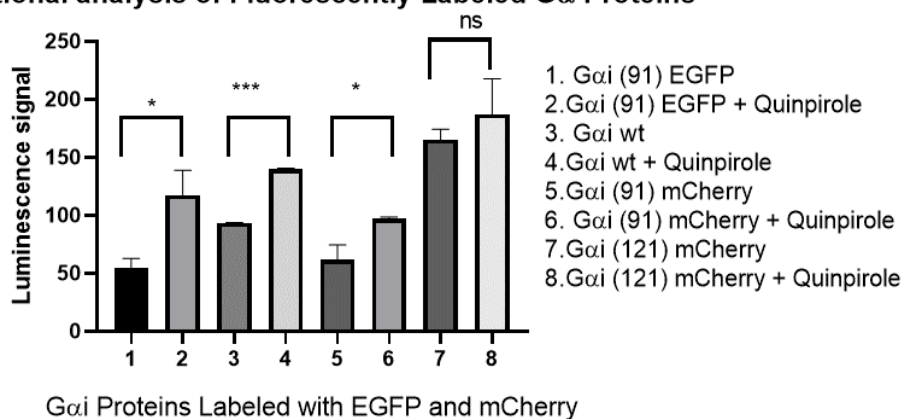


Figure 3. 1: cAMP-Glo Assay results of $G\alpha$ proteins labeled with EGFP and mCherry fluorophores. (Unpaired T-test applied for determination of the luminescence difference after ligand treatment, $G\alpha$ 91 EGFP p value: $0.0323 < 0.05$,

Gα 91 mCherry p value: 0.0347<0.05, Gα wild type p value: 0.0003<0.05, Gα 121 mCherry p value: 0.4627>0.05)

As mentioned in Chapter 1, Gαi proteins inhibit cAMP production in the cell and Gαs proteins are cAMP stimulatory proteins. The principle of cAMP-Glo Assay Kit is based on detection of cAMP via luminescent signal, which has an inverse proportion with cAMP concentration in the cell. Thus, according to the assay principle and Gαi protein activity, luminescence signal increase was expected due to reduced cAMP concentration up on Gi copled GPCR stimulation via an agonist ligand, Quinpirole . The assay was performed with the wild-type Gαi control group, and luminescence increase was compared.

Before Quinpirole treatment of the cells transfected with Gαi plasmids, Forskolin treatment was applied to increase basal cAMP concentration to observe Gαi effect much clearly. After ligand treatments, assay protocol was performed, and luminescence measurements were taken. T-test was applied to the groups that include cells transfected with the same plasmids for statistical analysis. Functionality of Gαi 91 EGFP and mCherry plasmid luminescence increase after Quinpirole treatment shows the functionality of these plasmids compared to only Forskolin treated group. However, Gαi 121 mCherry plasmid luminescence increase between Quinpirole treated and only forskolin treated group was not found significant according to T-test. The reason for this observation could be the constitutive activity of Gαi 121 mCherry protein due to a conformational change that could arise because of the tagging possition and maturation time of mCherry. (See Appendix E)

3.2 Confirmation of Membrane localization of G α Proteins and FRET Control Groups with Membrane Targeted Organelle Marker

Biological activity controlled EGFP and mCherry labeled G α i and G α s plasmids were transiently transfected into 60.000 N2A cells (for 35mm glass dishes 300 ng DNA was used). Since G α i 121 mCherry protein showed constitutive function this construct was not used in further experiments. Instead, EGFP labeled version of this construct was used. The expected cellular membrane expression pattern of G α proteins was validated by using plasma membrane marker Gap43 labeled with EGFP and mCherry fluorophores. Gap43 protein is a membrane-targeted protein (Huang *et al.*, 2015). Gap43 was labeled with EGFP and mCherry fluorescent protein by a former student of our laboratory Mr. Hüseyin Evci. Furthermore, a positive FRET control group with Gap43 mCherry (RGSLVPR) EGFP prepared by Mr. Hüseyin Evci was used during this thesis work (Albertazzi *et al.*, 2009).

Confocal microscope localization validation experiments showed G α i and G α s fusion proteins and Gap43 control group were located at the plasma membrane. Confocal images can be seen from Figure 3.2, Figure 3.3, Figure 3.4 and Figure 3.5.

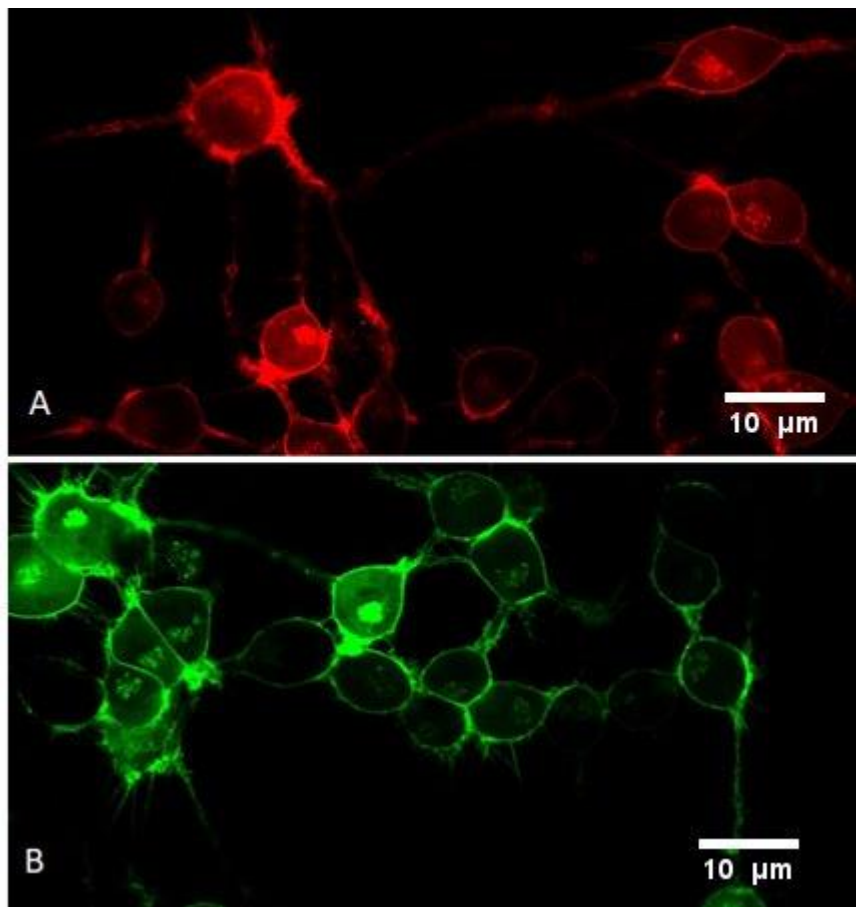


Figure 3. 2: Membrane targeted organelle marker Gap43 sequence labeled with A) mCherry and B) EGFP. mCherry protein samples were excited with 583 nm for 1000 ms, 100 % light intensity. EGFP protein samples excited with 482 nm for 1000 ms with 50 % light intensity, 63x and NA 1.4. objective was used for both channels.

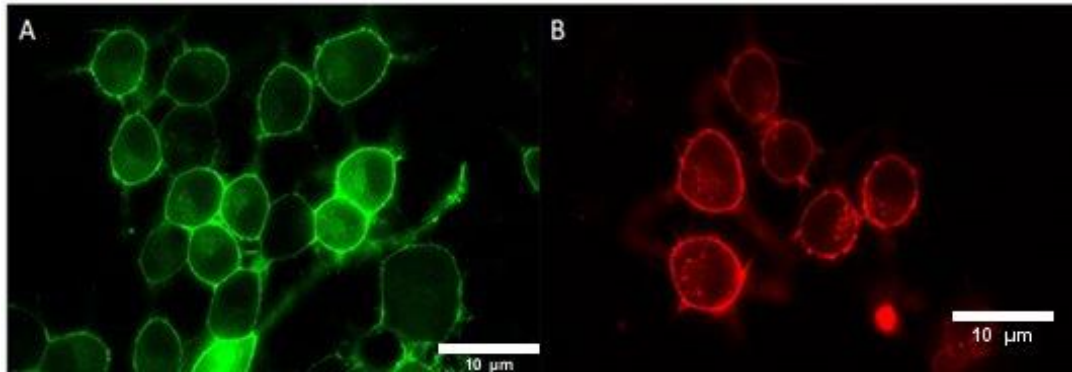


Figure 3. 3: Fluorescent protein labeled *Gas* $\Delta 73-85$ protein visualization. A)EGFP labeled *Gas* $\Delta 73-85$ protein, and B) mCherry labeled *Gas* $\Delta 73-85$ protein visualization. EGFP protein samples excited with 482nm for 1000 ms with intensity 50 %, 63x and NA 1.4. mCherry protein samples excited with 583nm, intensity 100% , 63x and NA 1.4.

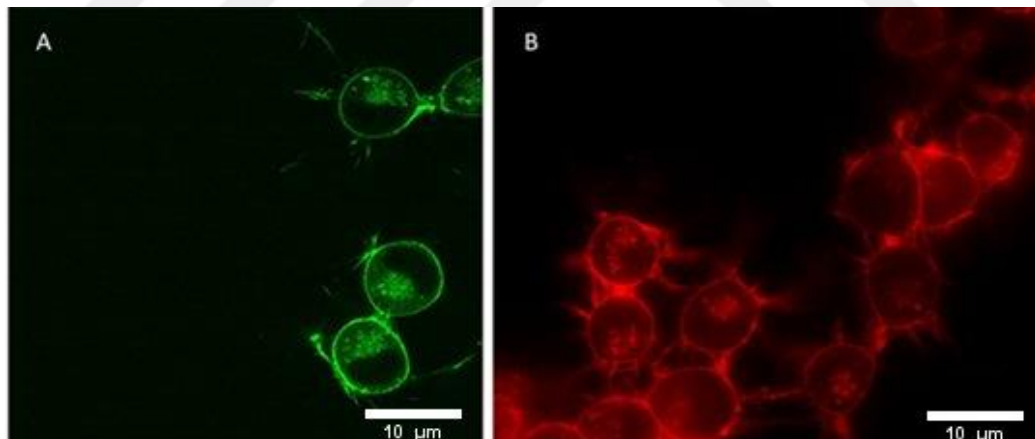


Figure 3. 4: Fluorescent protein labeled *Gai* 91 protein visualization. A)EGFP labeled *Gai* 91 protein, and B) mCherry labeled *Gai* 91 protein visualization. EGFP protein samples excited with 482nm for 1000 ms with intensity 50% , 63x and NA 1.4. mCherry protein samples excited with 583nm for 500ms. with intensity 100%, 63x and NA 1.4.

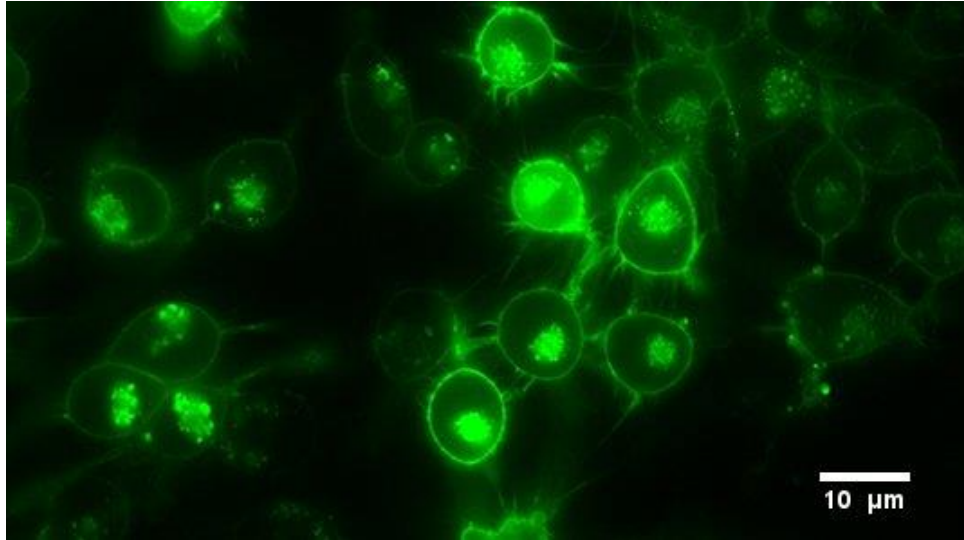


Figure 3. 5: EGFP Fluorescent protein labeled Gai 121 protein visualization. EGFP protein samples excited with 482 nm for 1000 ms with intensity 50 %, 63x and NA 1.4.

3.3 Investigation of Interaction Between Gai and Gas Proteins with FRET by Using Confocal Microscope

After localization confirmation of the EGFP and mCherry labeled Gα protein constructs, interactions between Gai and Gas proteins were investigated using Gai(91) and Gai(121) EGFP and Gas(73-85) mCherry plasmids. Constructs were transfected into N2a cells as 300 ng for 60.000 cells after the optimization process

for FRET study with spinning disc confocal microscopy imaging. 300 ng Gap43 EGFP and Gap43 mCherry plasmids were co-transfected to 60.000 N2a cells used as negative control group. In addition to this, Gap43 mCherry(RGSLVPR)EGFP fusion protein (Gap43 mCherry-L-EGFP) was used as the positive control group (Albertazzi *et al.*, 2009).

Imaging was performed with three sets of imaging tracks; FRET, EGFP, and mCherry. The reason for using EGFP and mCherry tracks is to calculate Spectral Bleed Through (SBT) that is used in PixFRET plug-in to calculate net FRET. The number of cells imaged in each set was approximately 20 . Images from each sets were SBT normalized and analyzed by using PixFRET plug-in of ImageJ. FRET efficiency ranges (1-10%, 11-20%, 21-30%, 31-40% and 41-50%) were determined and visualized via 5-ramp option with color code ruler and presented in Figure 3.6, Figure 3.7 and Figure 3.8.

Gap43 EGFP and Gap43 mCherry co-transfection group FRET efficiency value was calculated between 0-10% as shown in Figure 3.8 and Figure 3.9. The reason for this FRET value is long range (9-10 nm) random interactions between Gap43 EGFP and Gap43 mCherry fusion protein interactions at the N2a cell membrane. Gap43mCherry-L-EGFP positive FRET control group %FRET efficiency value was calculated between 3-30%. Gai(91) EGFP and Gas(73-85)mCherry fusion protein interaction FRET efficiency value was also calculated between 0-30% as presented in Figure 3.6 and Figure 3.9. Similarly, Gai(121) EGFP and Gas(73-85)mCherry fusion protein interaction FRET efficiency value was calculated as 0-30%.

It was found that up to 10% FRET efficiency, % pixel number of Gai – Gas FRET pairs are significantly different than Gap43 negative control group as shown in Figure 3.10($p < 0.05$), (See Appendix F). This result indicates, a physical interaction between Gai and Gas as close as the positive control where the donor and the acceptor are physicaly linked to each other.

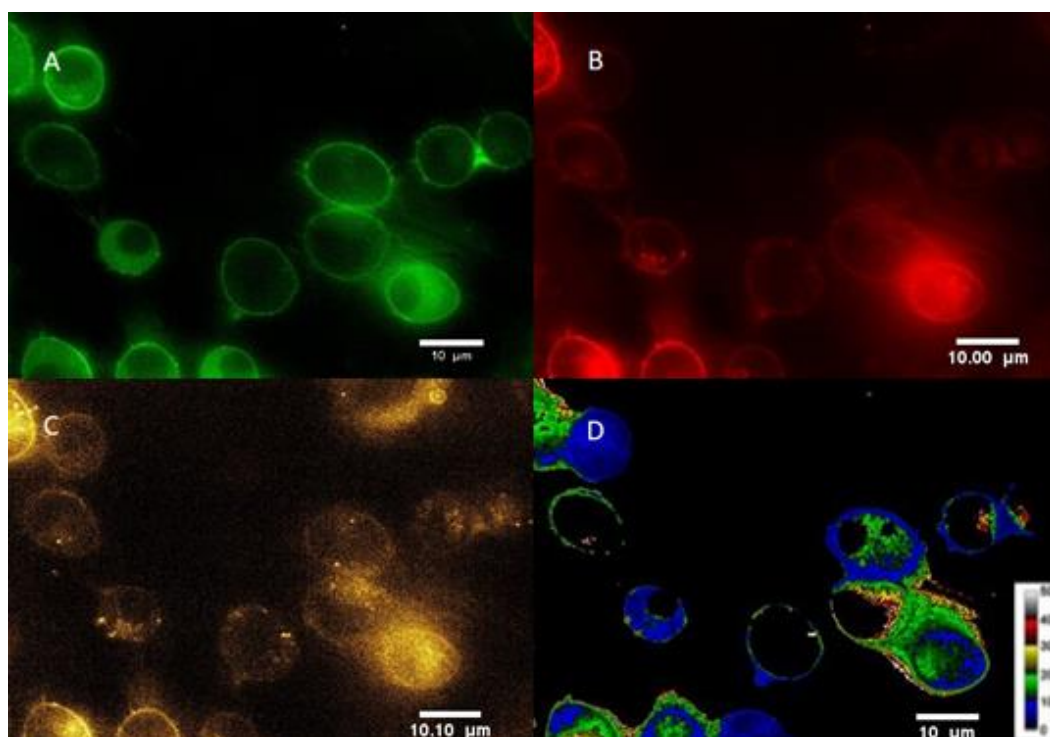


Figure 3. 6: Confocal FRET imaging of Gai(91)-EGFP and Gas(73-85)-mCherry. 300ng of Gai(91)-EGFP and Gas(73-85)-mCherry co transfected into N2a cell line. A) Image of EGFP channel; channel excitation at 482 nm with exposure time 0.5 s and intensity 50%. B) Image of mCherry channel; channel excitation at 586 nm with exposure time 1s and intensity 100%. C) Image of FRET channel; channel excitation at 482 nm with exposure time 0.5 s and intensity 50%. D) FRET efficiency in color scale; blue ,green, yellow,red and whire colors represent 0-10%, 11-20%, 21-30%, 31-40%, and 41-50% efficiencies of FRET, respectively.

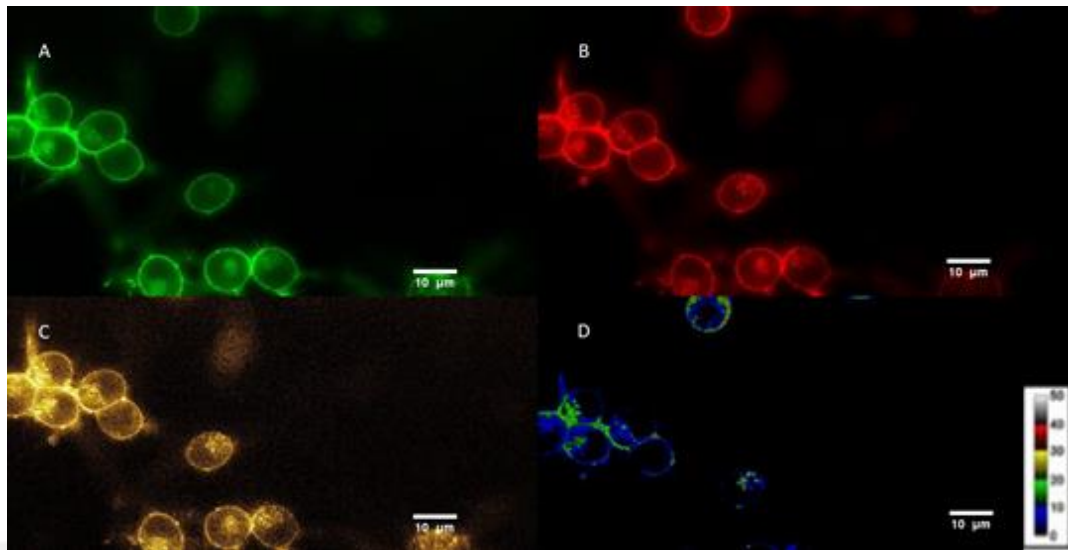


Figure 3. 7: Confocal FRET imaging of Gai(121)-EGFP and Gas(73-85)-mCherry. 300ng of Gai(121)-EGFP and Gas(73-85)-mCherry co transfected into N2a cell line. A) Image of EGFP channel; channel excitation at 482 nm with exposure time 0.5 s and intensity 50%. B) Image of mCherry channel; channel excitation at 586 nm with exposure time 1s and intensity 100%. C) Image of FRET channel; channel excitation at 482 nm with exposure time 0.5 s and intensity 50%. D) FRET efficiency in color scale; blue ,green, yellow,red and whire colors represent 0-10%, 11-20%, 21-30%, 31-40%, and 41-50% efficiencies of FRET, respectively.

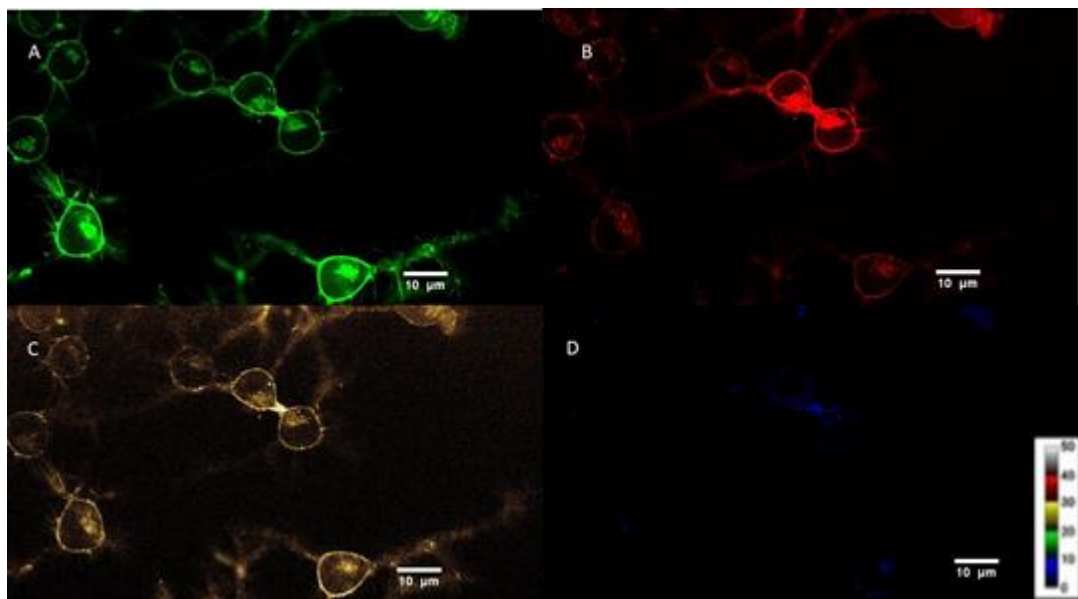


Figure 3. 8: Confocal FRET imaging of Gap43 EGFP and Gap43-mCherry. 300ng of Gap43 EGFP and Gap43mCherry co transfected into N2a cell line. A) Image of EGFP channel; channel excitation at 482 nm with exposure time 0.5 s and intensity 50%. B) Image of mCherry channel; channel excitation at 586 nm with exposure time 1s and intensity 100%. C) Image of FRET channel; channel excitation at 482 nm with exposure time 0.5 s and intensity 50%. D) Image of FRET efficiency; blue represents 0-10%, green 11-20%, yellow 21-30%, red 31-40%, and white 41-50% efficiency of FRET.

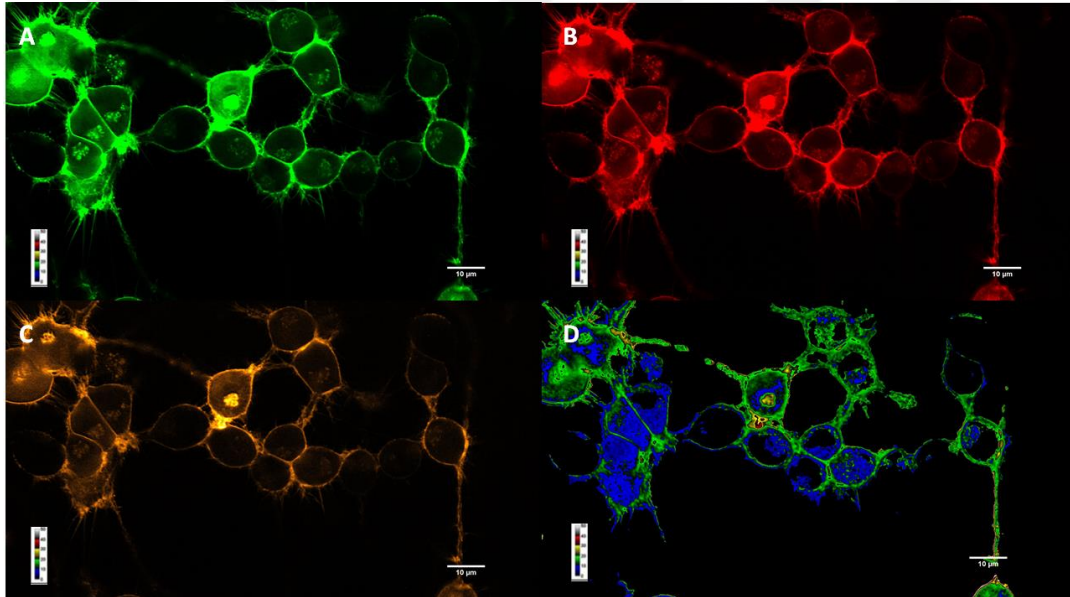


Figure 3. 9: Confocal FRET imaging of Gap43mCherry-L-EGFP. 300ng of plasmid transfected into N2a cell line. A) Image of EGFP channel; channel excitation at 482 nm with exposure time 0.5 s and intensity 50%. B) Image of mCherry channel; channel excitation at 586 nm with exposure time 1s and intensity 100%. C) Image of FRET channel; channel excitation at 482 nm with exposure time 0.5 s and intensity 50%. D) FRET efficiency in color scale; blue ,green, yellow,red and whire colors represent 0-10%, 11-20%, 21-30%, 31-40%, and 41-50% efficiencies of FRET, respectively.

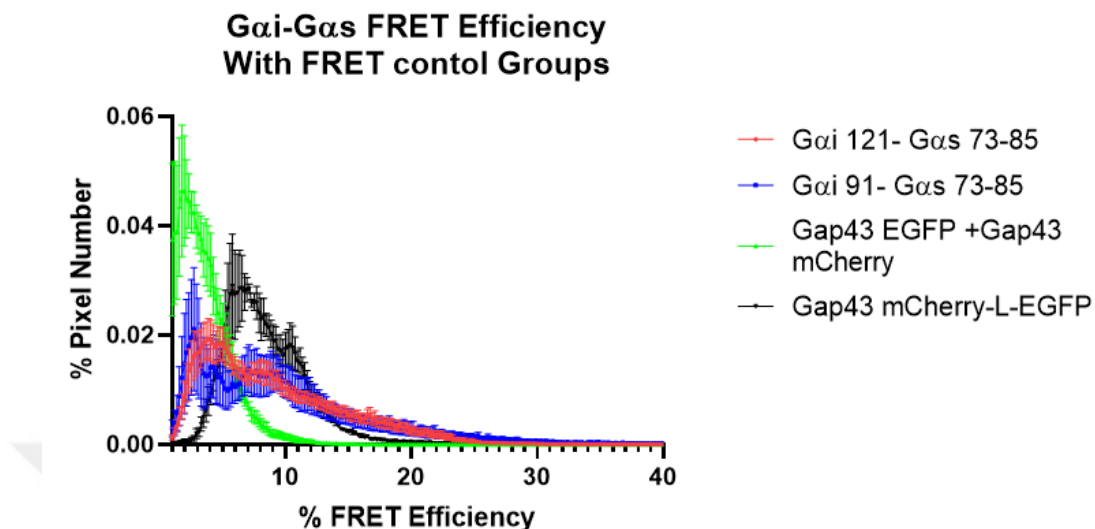


Figure 3. 10: Pix FRET analysis of confocal images. Green represents Gai(121)-EGFP and Gas(73-85)-mCherry, blue represents Gai(91)-EGFP and Gas(73-85)-mCherry and black represents Gap43 EGFP and Gap43-mCherry (negative control), Gap43mCherry-L-EGFP (positive control).

3.4 Investigation of Gai and Gas Interaction with FRET Technique Using Fluorescence Plate Reader

After confocal microscopy studies, Gai-Gas FRET pairs with Gai 121 EGFP- Gas (73-85) mCherry and Gai 91 EGFP - Gas (73-85) mCherry physical interactions were investigated by using a fluorescence plate reader to collect signals from a larger sample size. Ga constructs with fluorescent proteins and FRET control groups were transfected into N2A cells and seeded into black 96 well plates. Spectral measurements were taken following 450 nm EGFP excitation after the optimization process to reduce mCherry spectral bleed through. The area below the spectral signal was collected using Spectramax ID3 was calculated and normalized for all

measurements. Overall spectral region area was normalized to 1. The signal distribution plotted as line graph was presented in Figure 3.10-A. EGFP peak reduction and mCherry peak increase were expected when there is an energy transfer from donor fluorophore EGFP to acceptor fluorophore mCherry. As shown in Figure 3.10-A EGFP spectral peak (400-420 nm) of negative control group Gap43 EGFP + Gap43 mCherry was higher than positive control group Gap43 EGFP-L-mCherry and Gai-Gas FRET pairs. Furthermore, the mCherry peak of the negative control group does not exist since there were only random interactions between the donor and the acceptor fluorophores. Moreover, acceptor fluorophore mCherry spectral region (600-630nm) peaks were focused and analyzed with One way ANOVA since they had one categorical independent variable and one quantitative dependent variable with more than 3 groups. The negative FRET control group was found to be significantly different from positive FRET control, Gai 121 EGFP- Gas (73-85) mCherry and Gai 91 EGFP - Gas (73-85) mCherry FRET pairs ($p < 0.0001$). No significant differences were found between the positive FRET control group and Gai-Gas FRET pairs ($p > 0.05$). Furthermore, no significant difference was found between Gai 121 EGFP- Gas (73-85) mCherry and Gai 91 EGFP - Gas (73-85) mCherry FRET pairs ($p > 0.05$) (See Appendix G for overall data analysis).

Furthermore, to get rid of the cumulative results of the spectral combining of each well value and each dataset, acceptor (600-630nm) and donor (510-520nm) region ratio were calculated for normalized spectral area well measurements individually as shown in Figure 3.11. According to one way ANOVA analysis, no significant difference was found between Gai 121 EGFP- Gas (73-85) mCherry and Gai 91 EGFP - Gas (73-85) mCherry FRET pairs. On the other hand, Gai 121 EGFP- Gas (73-85) mCherry, Gai 91 EGFP - Gas (73-85) mCherry and Gap43 mCherry-L-EGFP FRET pairs were found significantly different from Gap43 EGFP, and Gap43 mCherry cotransfected group ($p < 0.05$). This experiment validates the interaction between Gai and Gas proteins.

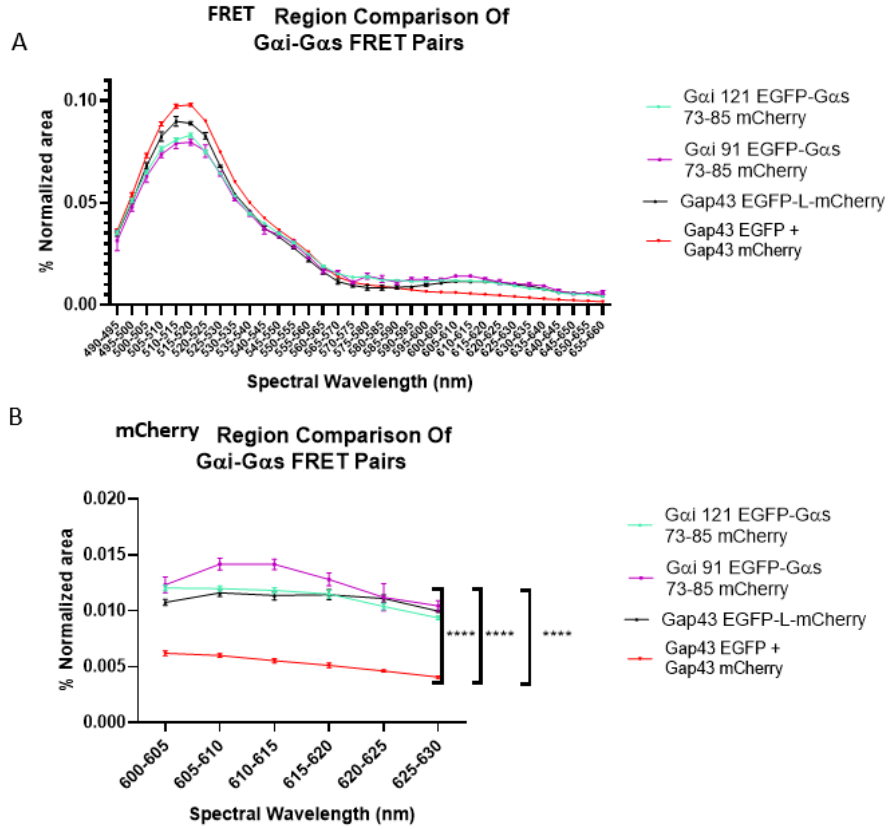


Figure 3. 11: Spectral area normalization of FRET spectrum of the Gai and Gas FRET pairs. A) Overall spectral region normalization, B) mCherry energy transfer region focused comparison between FRET control groups and Gai and Gas FRET pairs (Gai 121 EGFP-Gas 73-85 mCherry, Gai 91 EGFP-Gas 73-85 mCherry , Gap43 mCherry-L-EGFP, and Gap43 EGFP + Gap43mCherry presented as blue, pink, black and red, respectively. One way ANOVA used as statistical analysis. Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry $p < 0.0001$, Gai 91 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry $p < 0.0001$ and Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43 mCherry $p < 0.0001$, there are no significant difference between Gai 91 EGFP-Gas 73-85 mCherry, . Gai 121 EGFP-Gas 73-85 mCherry and Gap43 EGFP-L-mCherry $p > 0.05$).

Acceptor/Donor Peak Area Ratio Comparison Of G α i-G α s FRET Pair Spectrum

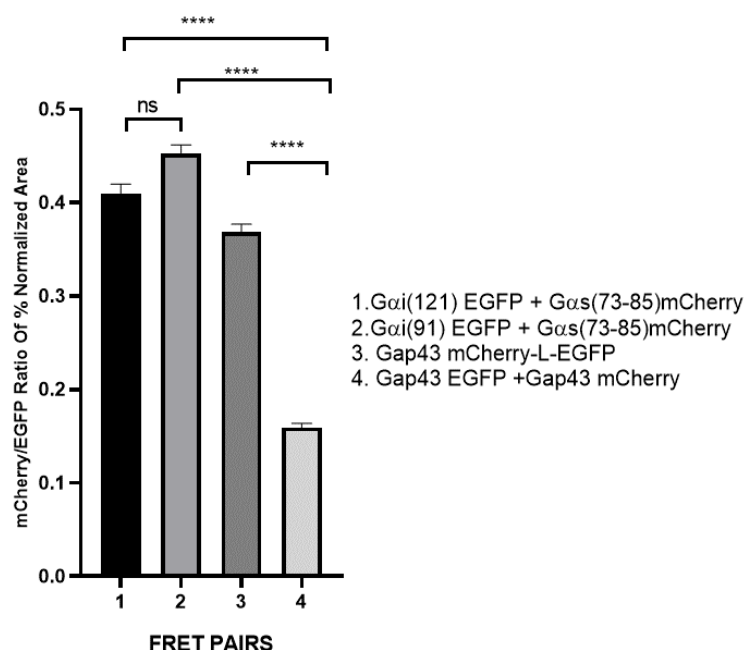


Figure 3. 12: Acceptor / Donor ratio of normalized spectrum of G α i-G α s and FRET control groups. (One way ANOVA applied results for groups; G α i 121 EGFP-G α s 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry $p < 0.05$, G α i 91 EGFP-G α s 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry $p < 0.05$ and Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43 mCherry $p < 0.05$ respectively.)

After these results, the study was continued using G α i (121) EGFP-G α s (73-85) mCherry FRET pair due to results that were given under Future Studies section.

3.5 Preparation and Sequence Analysis of G α i and G α s Specific Minigenes

G α protein-specific minigenes mainly include last 11 amino acids of specific G α protein and blocks receptor binding sites to inhibit G α protein GPCR interaction competitively. As it was mentioned in Chapter 1, in this study, G α i and G α s specific minigenes were used to inhibit receptor binding.

In order to insert *Ga* protein-specific minigenes, proper cut sites were selected. Since minigene size is smaller than the Multiple Cloning Site of the pcDNA3.1(-), restriction control with double digestion was not considered as an option. Thus, the PstI double cutter was used as control digestion. The diagram of restriction sites is depicted in Figure 3.13. For this strategy, primers design to delete PstI cut site inside the Multiple Cloning Site of the pcDNA3.1(-). For *Gai* minigene *Nhe*I and *Eco*RI cut sites , for *Gas* minigene *Nhe*I and *Hind*III cut sites selected.

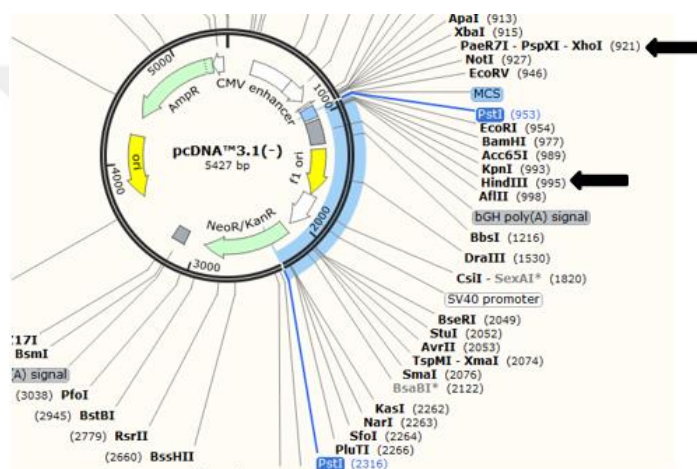


Figure 3. 13: Restriction sites for PstI double cutter for pcDNA 3.1(-)

For *Gai* and *Gas* minigenes, PCR mixture and optimal annealing conditions were set to 55 °C for Polymerase Chain Reaction (PCR). PCR product size was validated by loading 2X agarose gel and performing electrophoresis as presented in Figure 3.14A and Figure 3.15 A gel images. *Gai* and *Gas* minigene PCR amplicants size were found to be 66 bp as expected. No template control band from *Gas* minigene reaction was due to two reasons. The first reason was that the primers had high GC content and the second reason was that primers had overlapping regions.

After restriction, ligation, and transformation protocol, colonies were grown from agar plates inoculated into LB. Isolated plasmids were then cut by using PstI and

empty pcDNA3.1(-) was used as a negative control. From empty pcDNA3.1(-) and false-positive colony plasmids, it is expected that plasmid piece drop with 1363bp size and positive colonies were expected to be only linearize .

As expected, both G α i minigene and G α s minigene positive plasmids were linearized by comparing both empty pcDNA3.1(-) cut with Pst1 as a double cutter and empty pcDNA3.1(-) cut with HindIII as single cutter as presented from Figure 3.14 C and Figure 3.15 C gel images. After sequencing and analyzing the sequence results, no mutation was detected for both minigene samples.

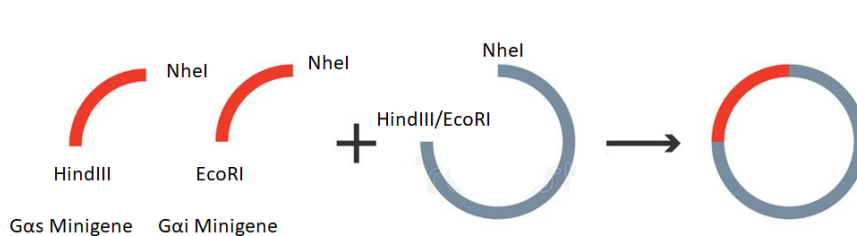


Figure 3. 14: G α i and G α s minigene cloning scheme.ö m56

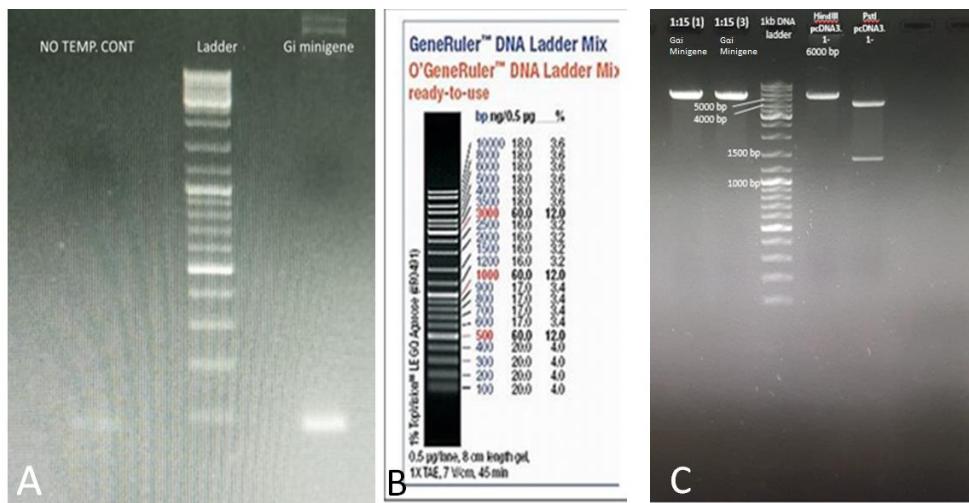


Figure 3. 15: G α i minigene cloning agarose gel results. A) PCR amplification B) Generuler DNA Ladder Mix map and C) Control restriction images. 1X Agarose gel

run with 110 V for 30 minutes for control restriction. For PCR products, 2X Agarose gel ran for 100 V for 45 minutes.

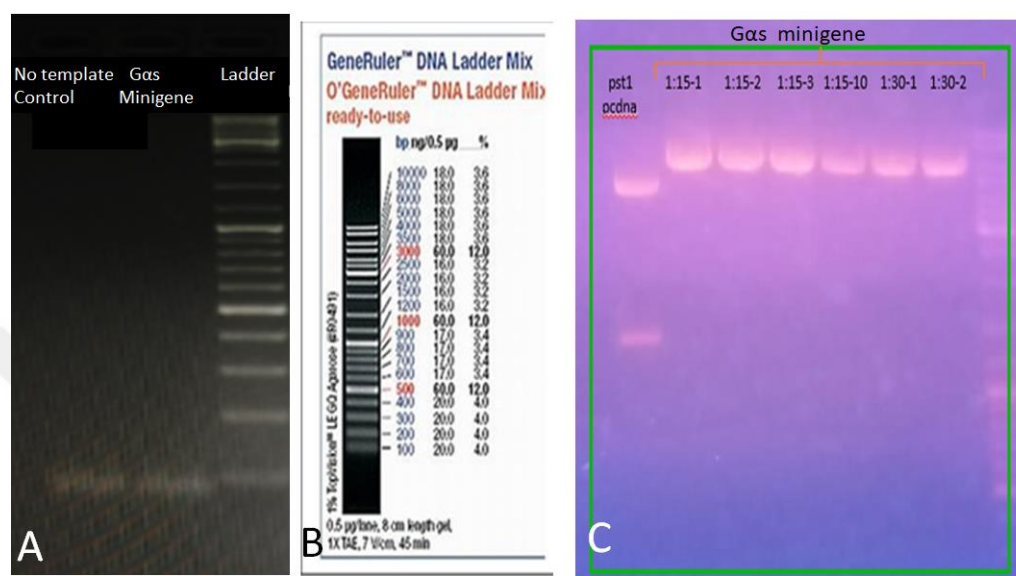


Figure 3. 16: Gas minigene cloning agarose gel results. A) PCR amplification, B) Generuler DNA Ladder Mix map, and C) Control restriction images. 1X Agarose gel run with 110 V for 30 minutes for control restriction. For PCR products, 2X Agarose gel ran for 100 V for 45 minutes.

3.6 Investigation of the Receptor Dependency of Gai and Gas Interaction by Using Minigenes

3.6.1 Investigation by Using Confocal Microscopy

After detection and validation of Gai-Gas interaction, receptor dependency of this interaction was investigated by using Gai and Gas specific minigene expression. Since there was no significant difference between Gai(121)EGFP-Gas(73-85)mCherry and Gai(91)EGFP-Gas(73-85)mCherry FRET efficiency,

Gai(121)EGFP-Gas(73-85)mCherry FRET pair was selected for the minigene experiment.

Gai(121)EGFP-Gas (73-85)mCherry with Gai and Gas minigenes, Gai(121)EGFP-Gas(73-85)mCherry and Gai(91)EGFP-Gas(73-85)mCherry groups were transfected into 100.000 N2a cells as 500 ng each. Since the DSD2 spinning disc confocal microscope from the lab was not working at this time, experiments were performed with Zeiss LSM 900, which is a laser scanning confocal microscope.

FRET efficiency calculations were performed using PixFRET plug-in. Gai(91)EGFP-Gas(73-85)mCherry FRET efficiencies calculated were found consistent with the previous results reported in this thesis using spinning disc confocal microscope. Membrane FRET efficiency signal was found in the 0-30% range as shown in Figure 3.17 and Figure 3.20. Gai(121)EGFP-Gas(73-85)mCherry and Gai(121)EGFP-Gas(73-85)mCherry + Gai and Gas minigene expressing group FRET efficiencies were found in the 0-35% range as shown in Figure 3.18, Figure 3.19 and Figure 3.20. The reason for this 5% increase for Gai(121)EGFP-Gas(73-85)mCherry group for laser scanning microscope data could be the transfection efficiency between spinning disc samples and laser scanning microscope samples. Low FRET efficiency calculated cell number could be more in spinning disc microscope samples.

In order to analyze the data, One-way ANOVA was used to compare 0-10% %FRET efficiency values between the two datasets. According to %FRET efficiency graph statistical analysis, all groups were found significantly different from each other ($p < 0.05$). Slight spectral shift to the left side of the Gai(121)EGFP-Gas(73-85)mCherry + Gai and Gas minigene group compared to Gai(121)EGFP-Gas(73-85)mCherry and Gai(91)EGFP-Gas(73-85)mCherry FRET pairs could mean that receptor interaction blocked Gai(121)EGFP-Gas(73-85)mCherry FRET pairs are capable of physical interaction (See Appendix H).

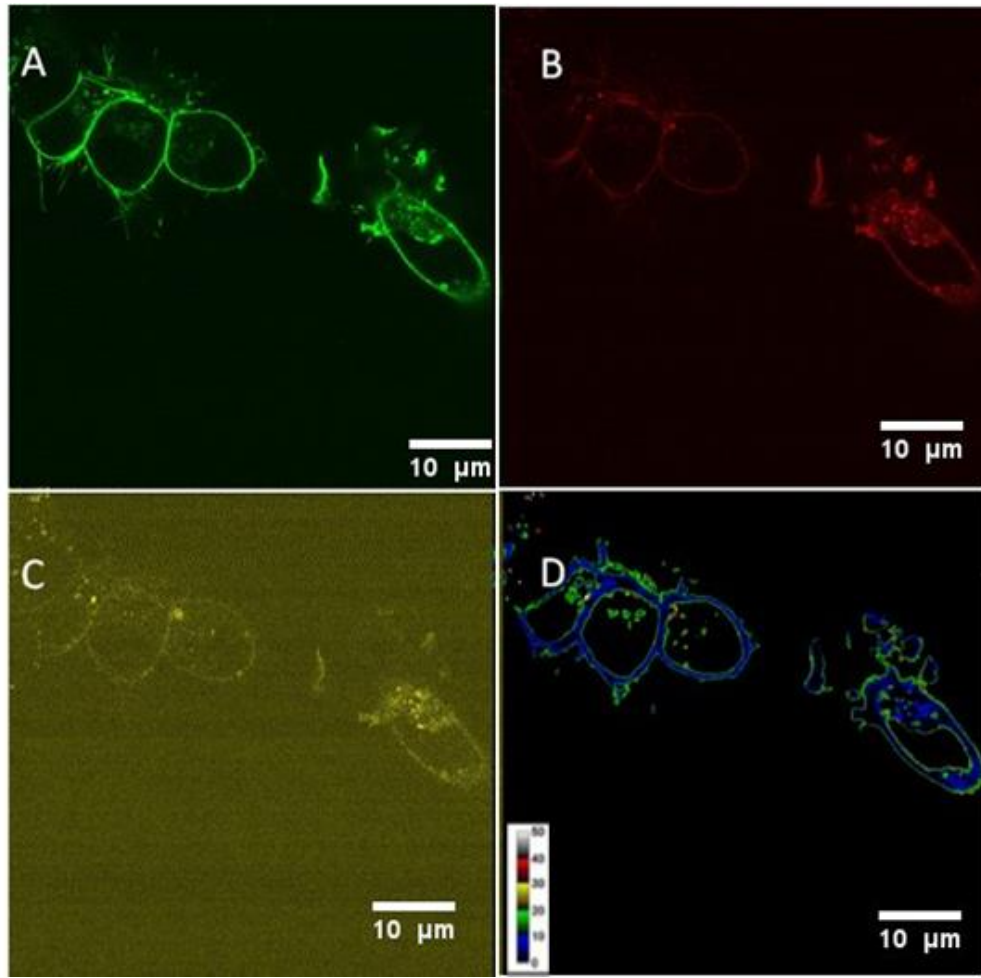


Figure 3. 17: LSM Airy scan Confocal FRET imaging of $G\alpha i(91)$ -EGFP and $G\alpha s(73-85)$ -mCherry. 500ng of $G\alpha i(91)$ -EGFP and $G\alpha s(73-85)$ -mCherry co-transfected into N2a cell line. A) Image of EGFP channel excited from 488nm with 800V and emission was taken from 510 nm by taking 490-575nm spectral region. B) Image of mCherry channel excited from 587 nm with 800V and emission was taken from 610 nm by taking 620-700 nm spectral region. C) Image of FRET channel excited from 488 nm with 800V and emission was taken from 610 nm by taking 620-700nm spectral region. D) FRET efficiency in color scale; blue ,green, yellow,red and whire colors represent 0-10%, 11-20%, 21-30%, 31-40%, and 41-50% efficiencies of FRET, respectively.

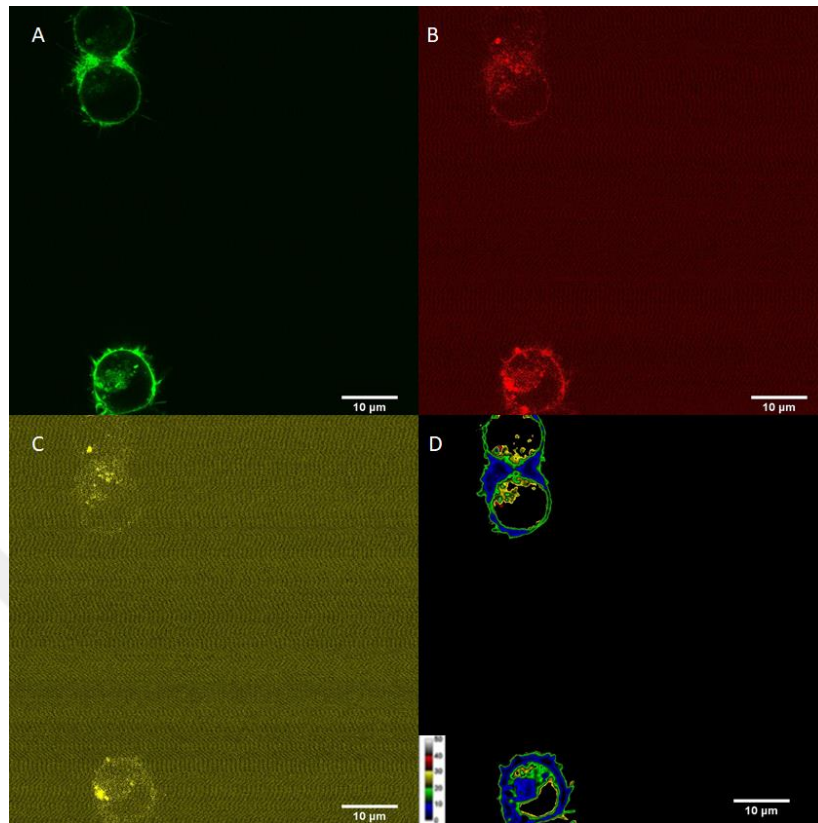


Figure 3. 18: LSM Airy scan Confocal FRET imaging of Gai(121)-EGFP and Gas(73-85)-mCherry. 500ng of Gai(121)-EGFP and Gas(73-85)-mCherry co-transfected into N2a cell line. A) Image of EGFP channel excited from 488nm with 800V and emission was taken from 510nm by taking 490-575nm spectral region. B) Image of mCherry channel excited from 587 nm with 800V and emission was taken from 610nm by taking 620-700nm spectral region. C) Image of FRET channel excited from 488 nm with 800V and emission was taken from 610nm by taking 620-700nm spectral region. D) FRET efficiency in color scale; blue ,green, yellow,red and whire colors represent 0-10%, 11-20%, 21-30%, 31-40%, and 41-50% efficiencies of FRET, respectively.

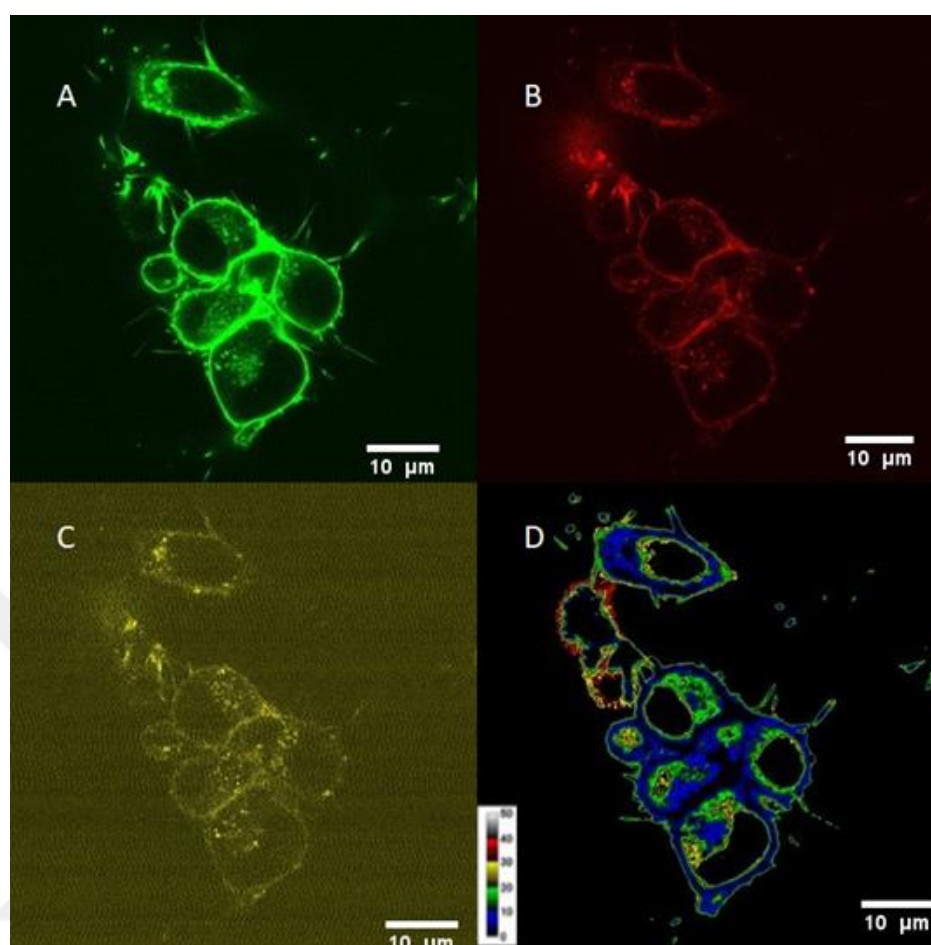


Figure 3. 19:LSM Airy scan Confocal FRET imaging of Gai(121)-EGFP and Gas(73-85)-mCherry + Gai and Gas minigenes. 500 ng of Gai(121)-EGFP, Gas(73-85)-mCherry with Gai and Gas minigenes co-transfected into N2a cell line. A) Image of EGFP channel excited from 488nm with 800V and emission was taken from 510nm by taking 490-575 nm spectral region. B) Image of mCherry channel excited from 587 nm with 800V and emission was taken from 610nm by taking 620-700nm spectral region. C) Image of FRET channel excited from 488 nm with 800V and emission was taken from 610nm by taking 620-700nm spectral region. D) FRET efficiency in color scale; blue ,green, yellow,red and whire colors represent 0-10%, 11-20%, 21-30%, 31-40%, and 41-50% efficiencies of FRET, respectively.

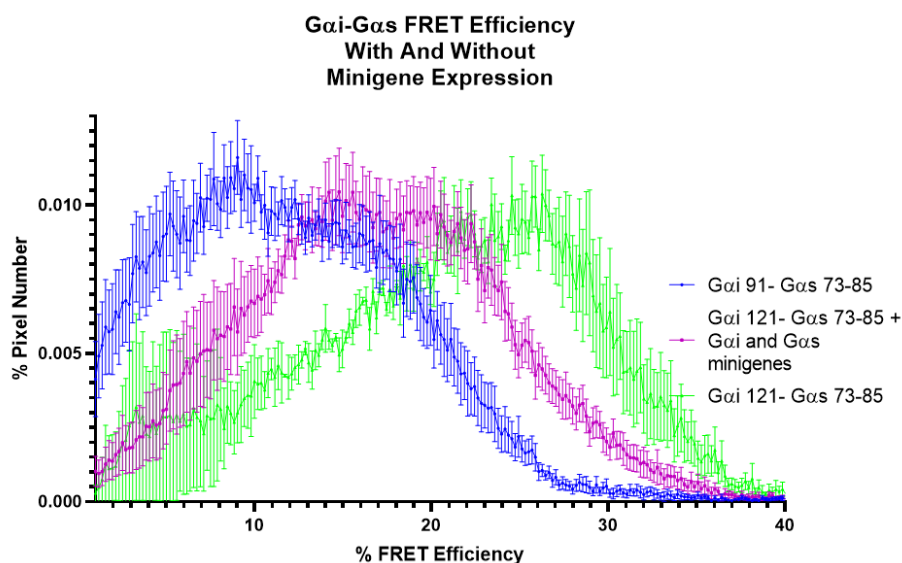


Figure 3. 20: Pix FRET analysis of confocal images. Green represents Gαi(121)-EGFP and Gαs(73-85)-mCherry, blue represents Gαi(91)-EGFP and Gαs(73-85)-mCherry and pink represents Gαi(121)-EGFP and Gαs(73-85)-mCherry + Gαi and Gαs minigene (One Way ANOVA performed to first 10% efficiency values, $p < 0.05$)

3.6.2 Investigation by Using Fluorescence Plate Reader

The classical GPCR-heterotrimeric G protein signaling model suggests that one GPCR binds one heterotrimeric G protein after ligand binding and secondary messenger system becomes activated. However, recent studies showed that GPCR oligomers, such as A2A-D2R heterotetramer, one heterotrimeric G protein α subunit can be shared by two GPCRs (Ferré *et al.*, 2016). Furthermore, studies showed that Ras family members, that belong to small G protein family, can form homodimers independent from their receptor (Inouye *et al.*, 2000). Thus, we investigated whether Gai-Gas physical interaction has receptor-independent property or not. For this purpose, Gai 121 EGFP-Gas 73-85 mCherry FRET pair interaction was further investigated in the presence of Gai and Gas specific minigenes. As it was stated in Chapter 1, minigenes block Gα protein interaction with GPCRs.

In order to collect signals from a larger sample size, Gai 121 EGFP-Gas 73-85 mCherry FRET pairs and Gai 121 EGFP-Gas 73-85 mCherry FRET pairs with Gai and Gas specific minigenes FRET groups were investigated with a fluorescence plate reader. The constructs were transfected into N2A cell line with FRET control groups as 500 ng for 100.000 cells. Gai 91 EGFP-Gas 73-85 mCherry FRET pair were not included in this experiment since earlier plate reader experiments gave no significant difference between Gai 91 EGFP-Gas 73-85 mCherry and Gai 121 EGFP-Gas 73-85 mCherry FRET pairs.

Spectral measurement was taken by using 450 nm excitation and taking emission signals in the 490-750 nm range for every 5 nm. From the raw forms of the measured spectral signal, area under the spectral signal line calculated and normalized. Acceptor peak region (600-630nm) was compared by using one-way ANOVA. No significant difference was found between Gai 121 EGFP-Gas 73-85 mCherry, Gai 121 EGFP-Gas 73-85 mCherry FRET pairs with Gai and Gas specific minigenes and Gap43mCherry-L-Gap43 EGFP ($p > 0.05$). Compared to Gap43 EGFP and Gap43 mCherry co-transfection group, Gai 121 EGFP-Gas 73-85 mCherry, Gai 121 EGFP-Gas 73-85 mCherry FRET pairs with Gai and Gas specific minigenes and Gap43mCherry-L-Gap43 EGFP were found to be significantly different as presented in Figure 3.21 ($p < 0.05$) (See Appendix I).

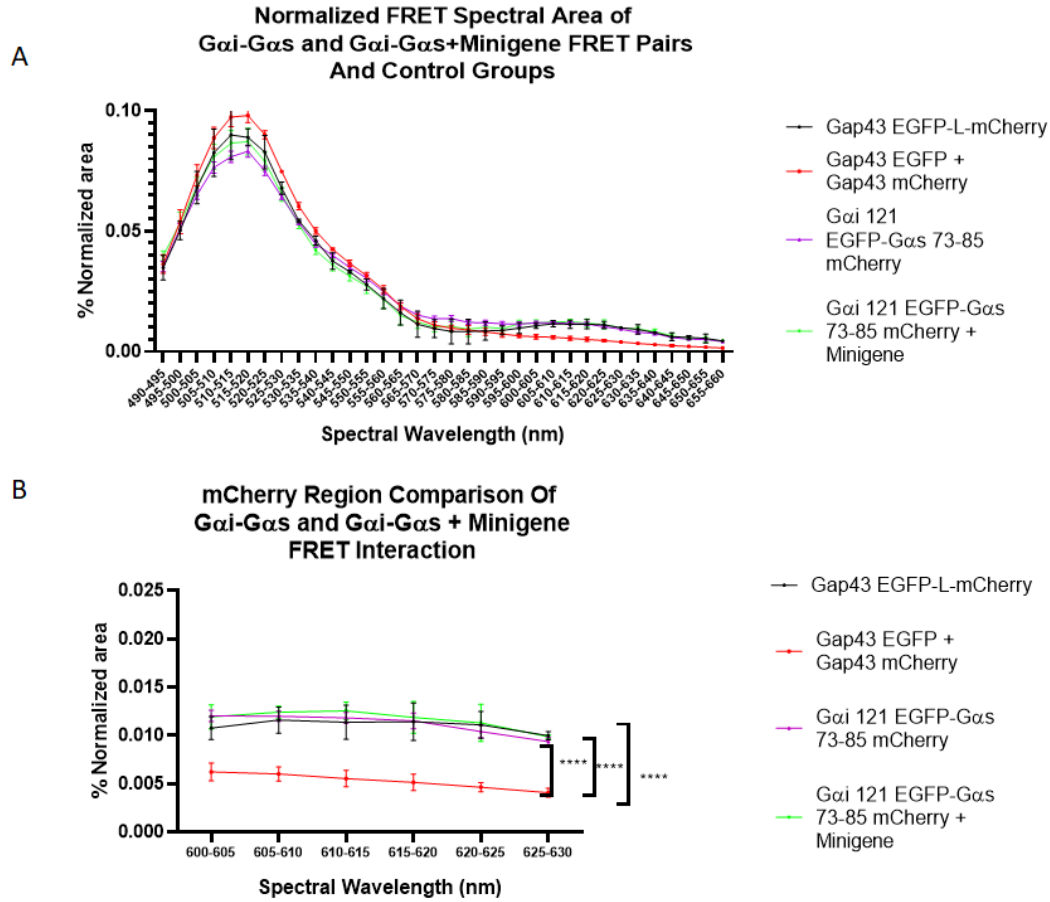


Figure 3. 21: Spectral area normalization of FRET spectrum of the Gai-G α s and minigene added Gai-G α s FRET pairs. A) Overall spectral region normalization, B) mCherry energy transfer region focused comparison between FRET control groups and Gai-G α s and minigene added Gai-G α s FRET pairs (One way ANOVA used as statistical analysis. Gai 121 EGFP-G α s 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry $p < 0.0001$, Gai 121 EGFP-G α s 73-85 mCherry + Minigene vs. Gap43 EGFP + Gap43 mCherry $p < 0.0001$ and Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43 mCherry $p < 0.0001$, there are no significant difference between Gai 121 EGFP-G α s 73-85 mCherry + Minigene vs. Gap43 EGFP, Gai 121 EGFP-G α s 73-85 mCherry and Gap43 EGFP-L-mCherry $p > 0.05$)

In order to avoid the combination of overall measurements for each wells; acceptor (600-630 nm) and donor (510-520 nm) region ratio was calculated for spectral area

normalized well measurements individually as presented in Figure 3.22. One-way ANOVA was applied to FRET pairs to be able to compare with each other. According to the analysis Gai 121 EGFP-Gas 73-85 mCherry FRET group was found significantly different from the Gai and Gas minigene expressing Gai 121 EGFP-Gas 73-85 mCherry FRET group ($p<0.05$). Statistical analysis of the fluorescence plate reader was found consistent with the laser scanning confocal microscope after statistical analysis (See Appendix I).

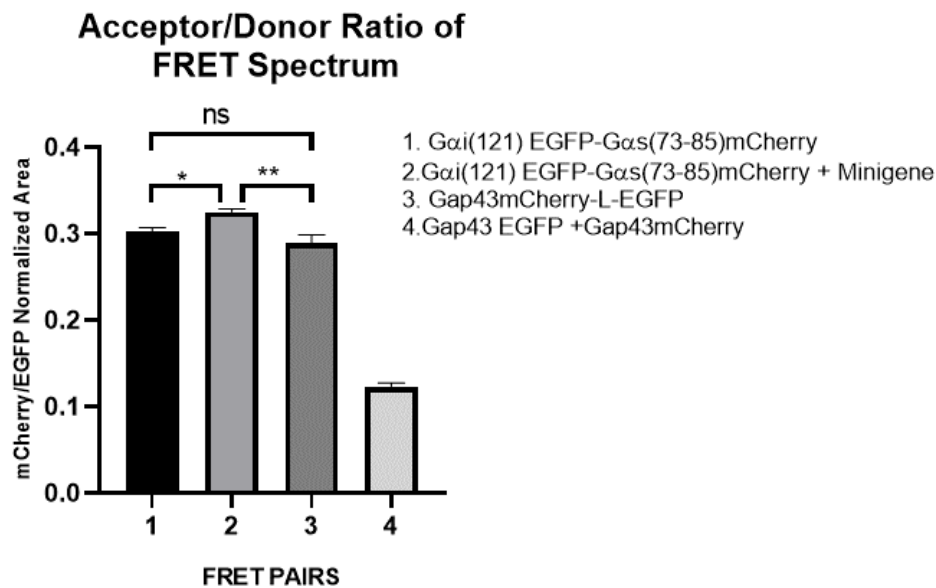


Figure 3. 22: Acceptor / Donor ratio of normalized spectrum of Gai-Gas and FRET control groups (One way ANOVA applied results for groups; Gai 121 EGFP-Gas 73-85 mCherry vs. Gai 121 EGFP-Gas 73-85 mCherry + Gai and Gas minigene $p<0.05$, Gai 121 EGFP-Gas 73-85 mCherry + Gai and Gas minigene vs + Gai and Gas minigene $p<0.05$, Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry $p<0.05$, Gai 91 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry $p<0.05$ and Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43 mCherry $p<0.05$ respectively).

3.7 Investigation of Gai and Gas Interaction with Agonist Treatment for D2R and A2R Receptor Signaling Pathway Activation by Using Fluorescence Plate Reader

After the investigation of receptor dependency, characteristics of Gai – Gas protein interaction for A2A -D2R GPCR heterotetramer structure were investigated as well. Gai 121 EGFP-Gas 73-85 mCherry FRET pair and Gai 121 EGFP-Gas 73-85 mCherry FRET pair with Gai and Gas specific minigenes FRET groups was transfected to N2A cell line with Gap43 FRET control groups as 500 ng for 100.000 cells. Before the measurement, 20 μ l 20 μ M CGS and 20 μ l 20 μ M Quinpirole were added on the cells and incubated at 37 °C at 5 % CO₂ for 20 minutes.

The measurements were performed with 450 nm excitation and 490-750nm emission spectrum with 5 nm steps. Spectral curve area was calculated and normalized for each dataset, as presented in Figure 3.23 A. Then, acceptor peak regions of the FRET pairs were compared by using 600-630 nm mCherry peak area. All Gai-Gas FRET pairs and Gap43 mCherry-L-EGFP positive control groups were found significantly different from the Gap43 EGFP and Gap43 mCherry co-transfected negative control as shown in Figure 3.23 B(p<0.05) (See Appendix J).

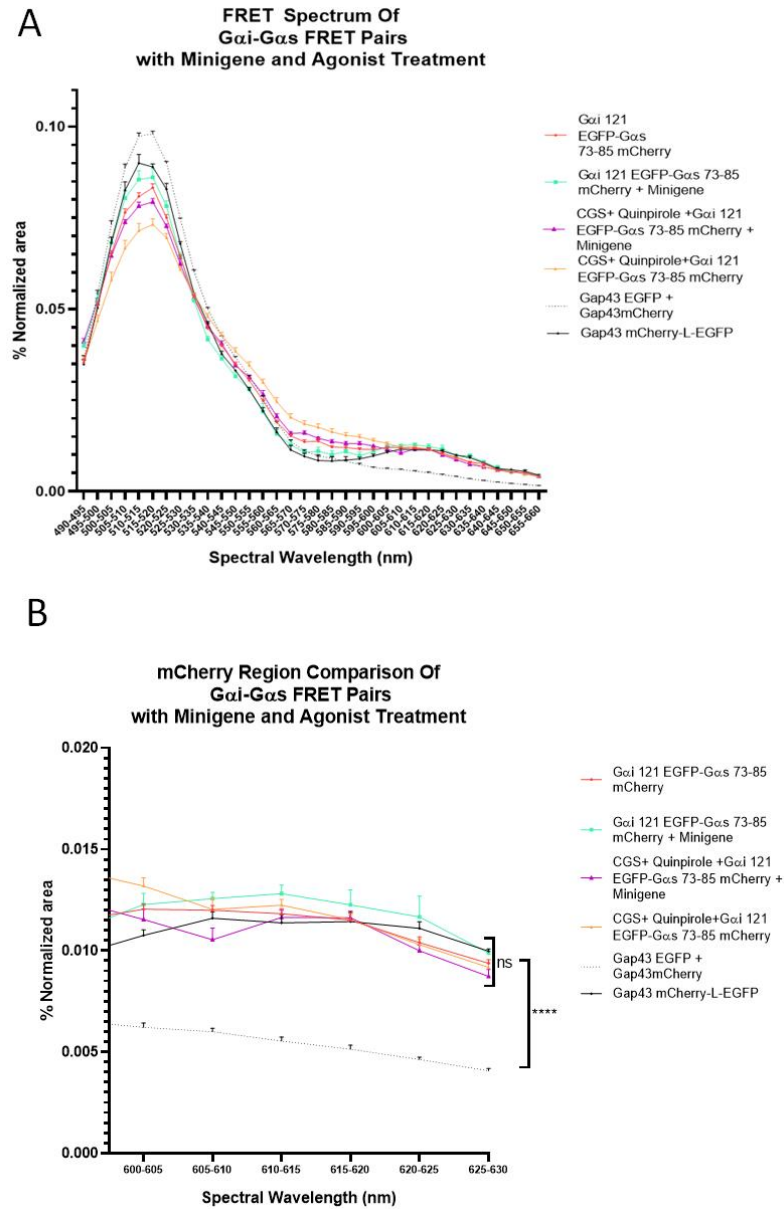


Figure 3. 23: Spectral area normalization of FRET spectrum of the Gai-Gas and minigene added Gai-Gas FRET pairs with and without CGS+Quinpirole treatment. A) Overall spectral region normalization, B) mCherry energy transfer region focused comparison between FRET control groups and Gai-Gas and minigene added Gai-Gas FRET pairs with and without CGS+Quinpirole treatment.

In order to avoid spectral combination of each dataset and each well, the spectral measurement from acceptor (600-630nm) and donor (510-520nm) peak region ratio was calculated for spectral area normalized well measurements individually as shown in Figure 3.24. CGS+Quinpirole treated Gai 121 EGFP-Gas 73-85 mCherry, no treatment applied Gai 121 EGFP-Gas 73-85 mCherry + Gai and Gas minigene and CGS+Quinpirole treated Gai 121 EGFP-Gas 73-85 mCherry + Gai and Gas minigene FRET group acceptor peak ratios were found significantly different from; Gai 121 EGFP-Gas 73-85 mCherry pair ($p < 0.05$). Furthermore, the ligand treatment was significantly increased FRET when compared CGS+Quinpirole Gai 121 EGFP-Gas 73-85 mCherry pair to minigene treated Gai 121 EGFP-Gas 73-85 mCherry ($p < 0.05$). However, there was no significant difference between CGS and Quinpirole treated Gai 121 EGFP-Gas 73-85 mCherry and CGS and Quinpirole treated Gai 121 EGFP-Gas 73-85 mCherry with Gai and Gas minigenes ($p > 0.05$)(See Appendix J).

According to the peak area ratio overall for Gai 121 EGFP-Gas, 73-85 mCherry FRET pairs with minigene and ligand treatments, both ligand treatment and minigene treatment increased the FRET individually. However, when both treatments were applied to Gai 121 EGFP-Gas 73-85 mCherry pair and compared to Gai 121 EGFP-Gas 73-85 mCherry with minigenes and CGS+Quinpirole treated Gai 121 EGFP-Gas 73-85 mCherry separately, no significant difference was found ($p > 0.05$). According to these results, there is receptor-independent interaction characteristics of Gai-Gas.

According to treatment characteristics of minigene expression, inhibition of GPCR- $G\alpha$ interaction is expected. Thus, increased peak ratio suggest GPCR independent dimer formation at the cell membrane possibly $G\alpha$ protein at GDP bound form. On the other hand, CGS and Quinpirole treatment activates A2A-D2R heterotetramer and $G\alpha$ protein separation from GPCR oligomers as GTP bound state. Increasing trend of mCherry peak of the ligand treated Gai-Gas FRET pair observed when compared to both minigene expressing Gai-Gas FRET pair and Gai-Gas FRET pair

with no treatment. According to these results, Gai-Gas interaction with A2A-D2R heterotetrameric structure was found more dominant when compared with minigene treated, possibly GDP bound, form.

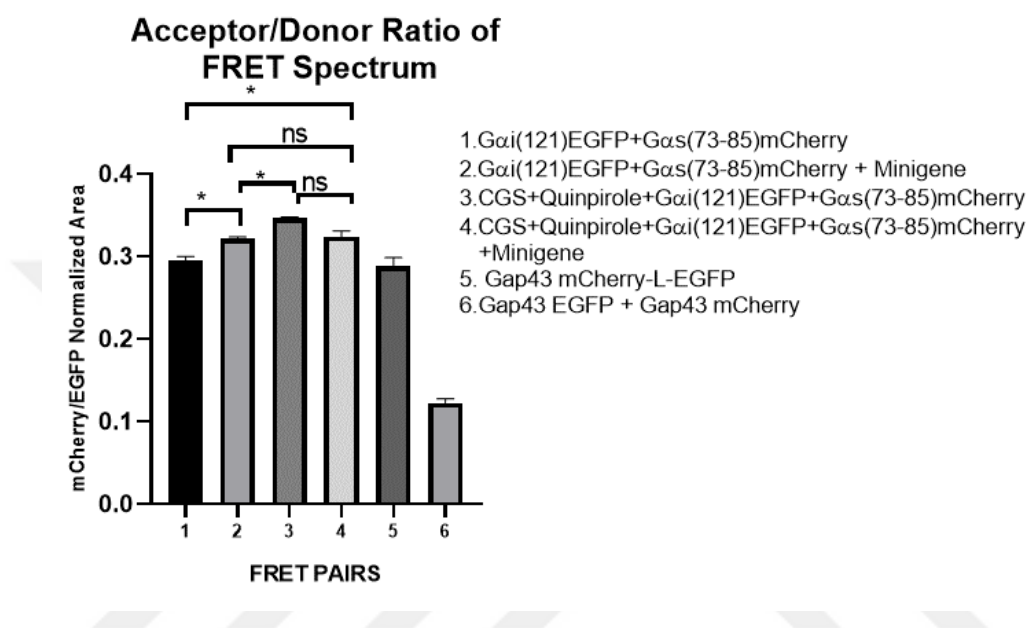


Figure 3. 24: Acceptor / Donor ratio of the normalized spectrum of Gai-Gas and FRET control groups with CGS and Quinpirole treatment. (According to one way ANOVA analysis: Gai 121 EGFP-Gas 73-85 mCherry vs. Gai 121 EGFP-Gas 73-85 mCherry + Gai and Gas minigenes $p < 0.05$; Gai 121 EGFP-Gas 73-85 mCherry vs. CGS+Quinpirole treated Gai 121 EGFP-Gas 73-85 mCherry $p < 0.05$; Gai 121 EGFP-Gas 73-85 mCherry vs. CGS+Quinpirole treated and minigene expressing Gai 121 EGFP-Gas 73-85 mCherry $p < 0.05$).

3.8 Future Studies

Since the FRET results obtained by spinning disc confocal microscopy data has several peaks, it suggests more than one group of interaction. This observation leads us to think fluorescently labeled Gα proteins can have different levels of closeness to each other, when the microscope images were examined it is apparent these different groups localized at different cellular locations. Following careful examination these locations are proposed as various organelles in the cell. In our future studies we plan to investigate various organelles and see if this hypothesis holds true. During this thesis period in order to have some preliminary data 3 different locations for 30 samples were selected and sample curves prepared using the FRET images. The data was kindly analyzed by Dr. Fatma Küçük Baloğlu using MATLAB and locations of curves predicted by a simulation. To analyze the signals, the same image datasets were used again and 30 cells were selected as a whole, not partial (Figure 3.25). Then, this data was fitted to simulated location curves.

The fitted data were analyzed by using One-Way ANOVA. According to statistical analysis results, no significant difference between Gαi(121)-Gαs(73-85) and Gαi(91)-Gαs(73-85) on membrane fitting was found ($p>0.05$). Furthermore, Gαi(121)-Gαs(73-85) was found to be significantly different than Gap43 EGFP and Gap43 mCherry co-transfection negative FRET pair ($p<0.05$). However, Gαi(91)-Gαs(73-85) FRET pair was not found to be significantly different than Gap43 EGFP and Gap43 mCherry co-transfection group ($p>0.05$) (See Appendix K).

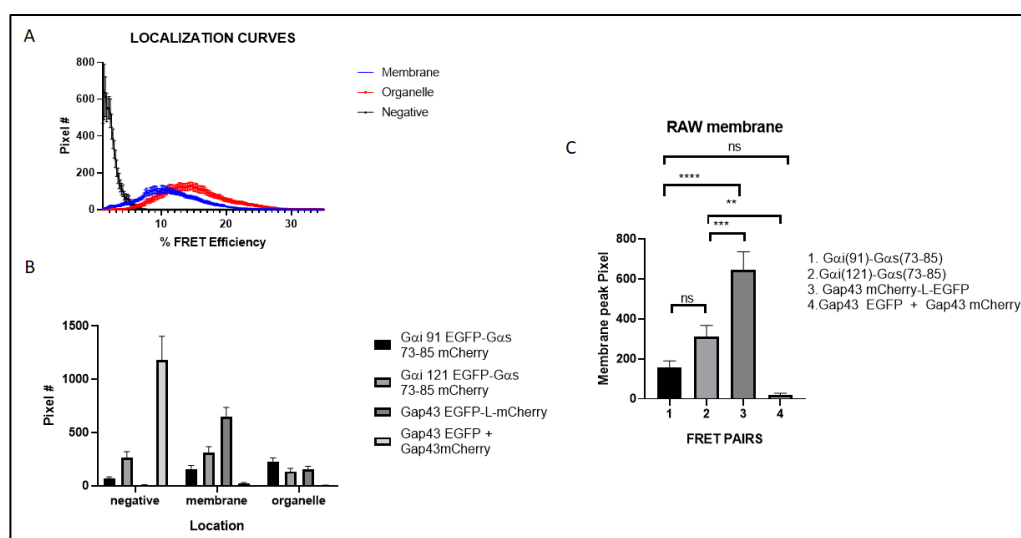


Figure 3. 25: Location-dependent curve fitting results with MATLAB. A) Model curves selected from different locations, B) Whole-cell selected FRET pair signal fit to model curves, C) Focusing on membrane signal.

3.9 Discussion

Gai and Gas proteins were found in theoretical interaction distance reported by Navarro *et al.* in 2016. In this study, for the first time, Gai and Gas protein interaction was shown using FRET technique. Furthermore, according to minigene expressed Gai and Gas FRET pair results, GPCR interaction interface blocked Gai and Gas interaction found significantly higher than basal Gai and Gas interaction. This finding suggests that there is a GPCR independent Gai and Gas interaction. On the other hand, results obtained following activation of A2A-D2R heterotetrameric structure suggests, possibly GTP bound Gai and Gas protein interaction is dominant in the cellular environment rather than GDP bound Gai and Gas protein interaction. Furthermore, these findings fit to GPCR-G protein interaction hypothesis claiming that both GPCR and the heterotrimeric G protein locates on the cell membrane separately until ligand activation.

The spectral unmixing and curve fitting study, that was applied on FRET efficiency calculated data sets, shows that 91st position used G α i and G α s protein interaction trend is more dominant in organelle regions than 121st position used G α i and G α s protein interactions.

Constitutively active K-Ras forms could act as oncogenes similarly studies with G α mutantas (especially G α 12) and chimeric G protein family proteins could be extremely useful for cancer studies. According to cAMP-Glo Assay results of ligand-activated and control group luminescence comparisons, using the G α i 121 mCherry construct we showed that some constructs made in this study could be constitutively active. Although these constructs could not be used in this study they could be valuable in further investigations of GTP bound form of G α i and G α s interaction characteristics such as interface, cellular FRET signal localization, and downstream pathways.

CHAPTER 4

CONCLUSION

Recent studies suggest that A1-A2A receptor heterotetramer and A2A-D2 receptor heterotetramer structures bring G_{ai} and G_{as} proteins within theoretical interaction distance (Ferre *et al.*, 2008; Navarro *et al.*, 2018). In this thesis, the interaction between G_{ai} and G_{as} proteins was investigated by FRET via both confocal microscopy and fluorescence plate reader measurements. The results from both studies suggest that these proteins interact with each other. Furthermore, the interaction dependency of receptor heterotetramers was investigated by blocking GPCR- G_{α} protein interaction via minigenes (Gilchrist *et al.*, 2002). The increase in the FRET signal from G_{ai} - G_{as} FRET pairs in the presence of minigenes suggests that there is a GPCR independent interaction with G_{α} proteins with possibly GDP bounded form. When focused on G_{ai} - G_{as} physical interaction with A2A-D2 receptor heterotetramer specific perspective using A2A receptor-ligand CGS and D2 receptor-specific ligand Quinpirole treatment, normalized acceptor spectral emission peak area over normalized donor spectral emission peak area ratio increased. This suggests that G_{ai} – G_{as} FRET pairs are interacting with GPCR unbound possibly GTP bounded form. This indicates that there are two different receptor-independent interaction types for G_{ai} - G_{as} proteins. These interaction types compared with each other by treating G_{ai} - G_{as} FRET pair with both minigene and A2A-D2 receptor ligands. Double treatment results were obtained statistically not different when compared with CGS+Quinpirole treatment and minigene treatment results from G_{ai} - G_{as} FRET pairs individually. This can be explained by the limitation of G_{α} protein binding to GPCR and competitively inhibiting to GTP exchange. According to this, two different G_{ai} - G_{as} GPCR independent interaction types were detected, and GTP bound interaction was found to be more dominant to

GDP bound interaction form. These findings fit to GPCR-G protein interaction hypothesis claiming that both GPCR and the heterotrimeric G protein locates on the cell membrane separately until ligand activation.



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APPENDICES

A. COMPOSITIONS OF SOLUTIONS

Table A. 1:DMEM High Glucose Composition

COMPONENT	CONCENTRATION (mg/L)
Amino Acids	
Glycine	30
L-Arginine hydrochloride	84
L-Cysteine 2HCl	63
L-Glutamine	580
L-Histidine hydrochloride-H ₂ O	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

Ingredients were dissolved in distilled H₂O. 20 g/L agar was added for LB Agar plate preparation. The pH of the prepared medium was adjusted to 7.0.

Phosphate Buffer Saline (PBS), 10X Stock Solution

11.5g Na_2HPO_4

80g NaCl

2g KCl

Components were dissolved into 1L deionize sterilized water and pH adjusted 7.4 for 1X PBS.

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

40mM Tris

20mM Acetic Acid

1mM EDTA

All components were dissolved in dH_2O .

Table A. 2: Composition of TFB I Solution

TFB1		
	250mL	
30 mM potassium acetate	0,74	g
10 mM CaCl ₂	1,25	mL of 2M
50 mM MnCl ₂	2,47	g
100 mM RbCl	3,023	g
15% glycerol	37,5	mL of 99%

Overall volume was completed to 100mL by using distilled H₂O and pH was adjusted to 5.8. Solution autoclaved or filtered with 0.45mm filter.

Table A. 3: Composition of TFB II Solution

TFB2		
	100mL	
10 mM MOPS or PIPES	0,335	g
75 mM CaCl ₂	3,75	mL of 2M
10 mM RbCl	0,12	g
15% glycerol	15	mL of 99%

Overall volume was completed to 100mL by using distilled H₂O and pH was adjusted to 5.8. Solution autoclaved or filtered with 0.45mm filter.

Induction Buffer (1X)

1X PBS with 100µM Ro 20-1724(4-(3-butoxy-4-methoxy-benzyl) imidazolidone) , Krebs Ringer Buffer and 500µM IBMX(3-isobutyl-1-methylxanthine).

B. MAMMALIAN EXPRESSION MAPS

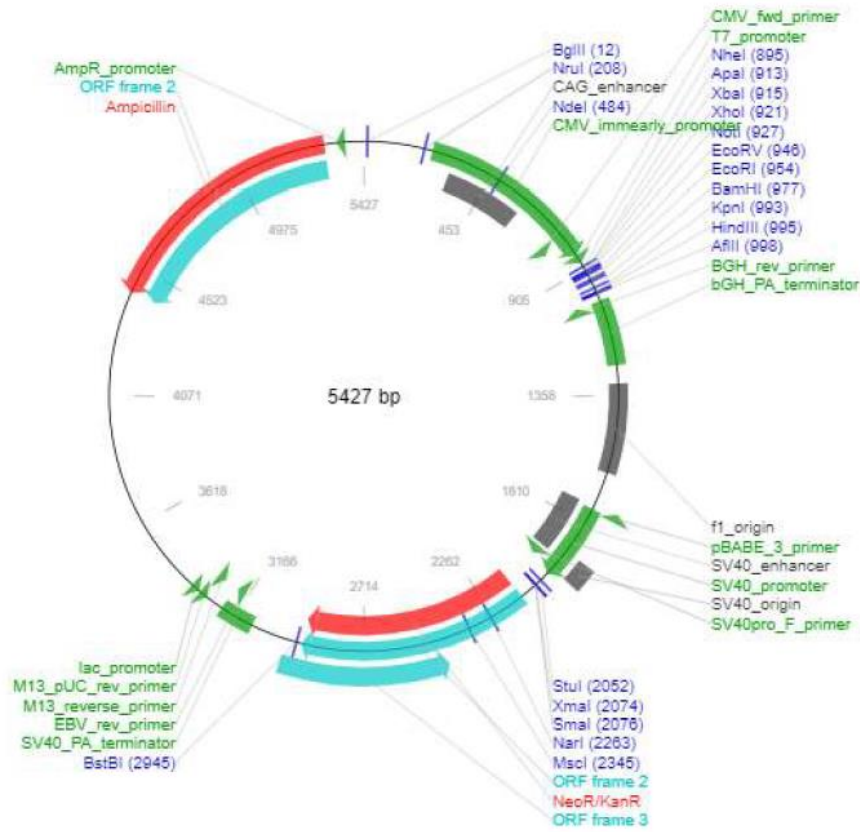


Figure B. 1: pcDNA3.1(-) expression vector map (taken from (*Addgene: Vector Database - PcDNA3.1(-)*, n.d.)

C. DESIGNED PRIMERS

Table C. 1: Minigene Cloning Primers

Gai Minigene Forward Primer	5'GTTGTTGTTCTCGAGGCCGCCACCATGGGAAT AAAAAATAATCTAAAAGA3'
Gai Minigene Reverse Primer	5'GTTGTTGTTAAGCTTTTATCCAAAGAGACCAC AA3'
Gas Minigene Forward Primer	5'GTTGTTGTTCTCGAGGCCGCCACCATGGGACA GCGCATGCACCTTCGTCAG3'
Gas Minigene Reverse Primer	5'AACAACAACAAGCTTTTATCCGAGCAGCTCG TACTGACGAAGG3'

D. FUSION PROTEIN CODING SEQUENCES

Gai 121 EGFP Coding Sequence:

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGT
AAGATGATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGC
GAGGTCAAGCTGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACA
ATTGTGAAGCAGATGAAAATTATCCATGAAGCTGGTTATTCAGAAGAG
GAGTGTAACAATACAAAGCAGTGGTCTACAGTAACACCATCCAGTCA
ATTATTGCTATCATTAGGGCTATGGGGAGGTTGAAGATAGACTTTGGTG
ACTCAGCCCGGGCGGATGATGCACGCCAACTCTTTGTGCTAGCTGGAGC
TGCTGAAGAAGGCTTTATGACTGCA**TC**TGGAGGAGGAGGATCTATGGT
GAGCAAGGGCGAGGAGCTGTTACACGGGGTGGTGCCCATCCTGGTCGA
GCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGG
CGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCAC
CACCGGCAAGCTGCCCGTGCCCTGGCCACCCTCGTGACCACCCTGACC
TACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAC
GACTTCTTCAAGTCCGCCATGCCC GAAGGCTACGTCCAGGAGCGCACCA
TCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGT
TCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACT
TCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACA ACTACA
ACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCA
AGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGC
TCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGC
TGCTGCCCGACAACCACTACCTGAGCACCCAGTCCAAGCTTAGCAAAG
ACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCG
CCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAG**TC**TGGAGGAG

GAGGATCTGAACTTGCTGGAGTTATAAAGAGATTGTGGAAAGATAGTG
GTGTACAAGCCTGTTTCAACAGATCCCGAGAGTACCAGCTTAATGATTC
TGCAGCATACTATTTGAATGACTTGGACAGAATAGCTCAACCAAATTAC
ATCCCGACTCAACAAGATGTTCTCAGAACTAGAGTGAAAACCTACAGGA
ATTGTTGAAACCCATTTTACTTTCAAAGATCTTCATTTTAAAATGTTTGA
TGTGGGAGGTCAGAGATCTGAGCGGAAGAAGTGGATTCATTGCTTCGA
AGGAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGACTACGACCTG
GTTCTAGCTGAAGATGAAGAAATGAACCGAATGCATGAAAGCATGAAA
TTGTTTGACAGCATATGTAACAACAAGTGGTTTACAGATACATCCATTA
TACTTTTTCTAAACAAGAAGGATCTTTTTGAAGAAAAAATCAAAAAGA
GCCCTCTCACTATATGCTATCAAGAATATGCAGGATCAAACACATATGA
AGAGGCAGCTGCATATATTCAATGTCAGTTTGAAGACCTCAATAAAAG
AAAGGACACAAAGGAAATATACACCCACTTCACATGTGCCACAGATAC
TAAGAATGTGCAGTTTGTGTTTTGATGCTGTAACAGATGTCATCATAAAA
AATAATCTAAAAGATTGTGGTCTCTTTTAG

Gai 121 mCherry Coding Sequence:

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGT
AAGATGATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGC
GAGGTCAAGCTGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACA
ATTGTGAAGCAGATGAAAATTATCCATGAAGCTGGTTATTCAGAAGAG
GAGTGTAACAATACAAAGCAGTGGTCTACAGTAACACCATCCAGTCA
ATTATTGCTATCATTAGGGCTATGGGGAGGTTGAAGATAGACTTTGGTG
ACTCAGCCCGGGCGGATGATGCACGCCAACTCTTTGTGCTAGCTGGAGC
TGCTGAAGAAGGCTTTATGACTGCACTGGAGGAGGAGGATCTATGGT
GAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCG

CTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGAT
CGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGACCGCCA
AGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCT
GTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCC
GACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGG
AGCGCGTGATGAACTTCGAGGACGGCGGGCGTGGTGACCGTGACCCAGG
ACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCG
GCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGG
GCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGA
AGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTAC
GACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGACGCTG
CCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACG
AGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACT
CCACCGGCGGCATGGACGAGCTGTACAAGTCTGGAGGAGGAGGATCTG
AACTTGCTGGAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAG
CCTGTTTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTGCAGCATA
CTATTTGAATGACTTGGACAGAATAGCTCAACCAAATTACATCCCGACT
CAACAAGATGTTCTCAGAACTAGAGTGAAAACCTACAGGAATTGTTGAA
ACCCATTTTACTTTCAAAGATCTTCATTTTAAAATGTTTGATGTGGGAGG
TCAGAGATCTGAGCGGAAGAAGTGGATTTCATTGCTTCGAAGGAGTGGC
GGCGATCATCTTCTGTGTAGCACTGAGTGACTACGACCTGGTTCTAGCT
GAAGATGAAGAAATGAACCGAATGCATGAAAGCATGAAATTGTTTGAC
AGCATATGTAACAACAAGTGGTTTACAGATACATCCATTATACTTTTTC
TAAACAAGAAGGATCTTTTTGAAGAAAAAATCAAAAAGAGCCCTCTCA
CTATATGCTATCAAGAATATGCAGGATCAAACACATATGAAGAGGCAG
CTGCATATATTCAATGTCAGTTTGAAGACCTCAATAAAAGAAAGGACA
CAAAGGAAATATACACCCACTTCACATGTGCCACAGATACTAAGAATG
TGCAGTTTGTGTTTTGATGCTGTAACAGATGTCATCATAAAAAATAATCT
AAAAGATTGTGGTCTCTTTTAG

Gai 91 EGFP Coding Sequence:

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGT
AAGATGATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGC
GAGGTCAAGCTGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACA
ATTGTGAAGCAGATGAAAATTATCCATGAAGCTGGTTATTCAGAAGAG
GAGTGTAACAATACAAAGCAGTGGTCTACAGTAACACCATCCAGTCA
ATTATTGCTATCATTAGGGCTATGGGGAGGTTGTCTGGAGGAGGAGGAT
CTATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCC
TGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCG
GCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCA
TCTGCACCACCGGCAAGCTGCCCCTGCCCTGGCCCACCCTCGTGACCAC
CCTGACCTACGGCGTGCAAGTGCTTCAGCCGCTACCCCGACCACATGAAG
CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGC
GCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGG
TGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCA
TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACA
ACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACG
GCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCG
TGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCC
CCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCAAGCTTAG
CAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGT
GACCGCCGCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCTGG
AGGAGGAGGATCTAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGA
TGCACGCCAACTCTTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATG
ACTGCAGAACTTGCTGGAGTTATAAAGAGATTGTGGAAGATAGTGGT
GTACAAGCCTGTTTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTG
CAGCATACTATTTGAATGACTTGGACAGAATAGCTCAACCAAATTACAT
CCCGACTCAACAAGATGTTCTCAGAACTAGAGTGAAAACCTACAGGAAT
TGTTGAAACCCATTTTACTTTCAAAGATCTTCATTTTAAAATGTTTGATG

TGGGAGGTCAGAGATCTGAGCGGAAGAAGTGGATTTCATTGCTTCGAAG
GAGTGGCGGCGATCATCTTCTGTGTAGCACTGAGTGACTACGACCTGGT
TCTAGCTGAAGATGAAGAAATGAACCGAATGCATGAAAGCATGAAATT
GTTTGACAGCATATGTAACAACAAGTGGTTTACAGATACATCCATTATA
CTTTTTCTAAACAAGAAGGATCTTTTTGAAGAAAAAATCAAAAAGAGC
CCTCTCACTATATGCTATCAAGAATATGCAGGATCAAACACATATGAAG
AGGCAGCTGCATATATTCAATGTCAGTTTGAAGACCTCAATAAAAGAA
AGGACACAAAGGAAATATACACCCACTTCACATGTGCCACAGATACTA
AGAATGTGCAGTTTGTGTTTTGATGCTGTAACAGATGTCATCATAAAAAA
TAATCTAAAAGATTGTGGTCTCTTTTAG

Gai 91 mCherry Coding Sequence:

ATGGGCTGCACGCTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGT
AAGATGATCGACCGCAACCTCCGTGAGGACGGCGAGAAGGCGGCGCGC
GAGGTCAAGCTGCTGCTGCTCGGTGCTGGTGAATCTGGTAAAAGTACA
ATTGTGAAGCAGATGAAAATTATCCATGAAGCTGGTTATTCAGAAGAG
GAGTGTAACAATACAAAGCAGTGGTCTACAGTAACACCATCCAGTCA
ATTATTGCTATCATTAGGGCTATGGGGAGGTTGTCTGGAGGAGGAGGAT
CTATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGT
TCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGT
TCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGA
CCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGG
ACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCA
CCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTC
AAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTG
ACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAG
CTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAG
ACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGC

GCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGG
CCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGT
GCAGCTGCCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCC
CACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGC
CGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTCTGGAGGAGGA
GGATCTAAGATAGACTTTGGTGACTCAGCCCGGGCGGATGATGCACGC
CAACTCTTTGTGCTAGCTGGAGCTGCTGAAGAAGGCTTTATGACTGCAG
AACTTGCTGGAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAG
CCTGTTTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTGCAGCATA
CTATTTGAATGACTTGGACAGAATAGCTCAACCAAATTACATCCCGACT
CAACAAGATGTTCTCAGAACTAGAGTGAAAACACTACAGGAATTGTTGAA
ACCCATTTTACTTTCAAAGATCTTCATTTTAAAATGTTTGATGTGGGAGG
TCAGAGATCTGAGCGGAAGAAGTGGATTCATTGCTTCGAAGGAGTGGC
GGCGATCATCTTCTGTGTAGCACTGAGTGACTACGACCTGGTTCTAGCT
GAAGATGAAGAAATGAACCGAATGCATGAAAGCATGAAATTGTTTGAC
AGCATATGTAACAACAAGTGGTTTACAGATACATCCATTATACTTTTTTC
TAAACAAGAAGGATCTTTTTTGAAGAAAAAATCAAAAAGAGCCCTCTCA
CTATATGCTATCAAGAATATGCAGGATCAAACACATATGAAGAGGCAG
CTGCATATATTCAATGTCAGTTTGAAGACCTCAATAAAAGAAAGGACA
CAAAGGAAATATACACCCACTTCACATGTGCCACAGATACTAAGAATG
TGCAGTTTGTTTTTGTATGCTGTAACAGATGTCATCATAAAAAATAATCT
AAAAGATTGTGGTCTCTTTTAG

Gas Δ 73-85 EGFP Coding Sequence:

ATGGGCTGCCTCGGGAACAGTAAGACCGAGGACCAGCGCAACGAGGA
GAAGGCGCAGCGTGAGGCCAACAAAAAGATCGAGAAGCAGCTGCAGA
AGGACAAGCAGGTCTACCGGGCCACGCACCGCCTGCTGCTGCTGGGTG
CTGGAGAATCTGGTAAAAGCACCATTGTGAAGCAGATGAGGATCCTGC
ATGTTAATGGGTTTAATGGAGAGGGCGGCGATGGTGAGAAGGCAACCA
AAGTGCAGGACATCAAAAACAACCTGAAAGAGGCGATTGAAACCATTG
TGGCCGCCATGAGCAACCTGGTGCCCCCGTGAGCTGGCCAACCCCG
AGAACCAGTTCAGAGTGGACTACATTCTGAGTGTGATGAACGTGCCTG
ACTTTGACTTCCCTCCCGAATTCTATGAGCATGCCAAGGCTCTGTGGGA
GGATGAAGGAGTGCGTGCCTGCTACGAACGCTCTGGAGGAGGAGGATC
TATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCT
GGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGG
CGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCAT
CTGCACCACCGGCAAGCTGCCCCTGCCCTGGCCACCCTCGTGACCACC
CTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGC
AGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGC
GCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGG
TGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCA
TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACA
ACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACG
GCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCG
TGCAGCTCGCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCC
CCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCAAGCTTAG
CAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGT
GACCGCCGCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCTGG
AGGAGGAGGATCTTCCAACGAGTACCAGCTGATTGACTGTGCCCAGTA
CTTCCTGGACAAGATCGACGTGATCAAGCAGGCTGACTATGTGCCGAG
CGATCAGGACCTGCTTCGCTGCCGTGTCCTGACTTCTGGAATCTTTGAG
ACCAAGTTCCAGGTGGACAAAGTCAACTCCACATGTTTGACGTGGGTG
GCCAGCGCGATGAACGCCGCAAGTGGATCCAGTGCTTCAACGATGTGA

CTGCCATCATCTTCGTGGTGGCCAGCAGCAGCTACAACATGGTCATCCG
GGAGGACAACCAGACCAACCGCCTGCAGGAGGCTCTGAACCTCTTCAA
GAGCATCTGGAACAACAGATGGCTGCGCACCATCTCTGTGATCCTGTTC
CTCAACAAGCAAGATCTGCTCGCTGAGAAAGTCCTTGCTGGGAAATCG
AAGATTGAGGACTACTTTCCAGAATTTGCTCGCTACACTACTCCTGAGG
ATGCTACTCCCGAGCCCGGAGAGGACCCACGCGTGACCCGGGCCAAGT
ACTTCATTCGAGATGAGTTTCTGAGGATCAGCACTGCCAGTGGAGATGG
GCGTCACTACTGCTACCCTCATTTCACCTGCGCTGTGGACACTGAGAAC
ATCCGCCGTGTGTTCAACGACTGCCGTGACATCATTACGCGCATGCACC
TTCGTCAGTACGAGCTGCTCTAG

Gas Δ 73-85 mCherry Coding Sequence:

ATGGGCTGCCTCGGGAACAGTAAGACCGAGGACCAGCGCAACGAGGA
GAAGGCGCAGCGTGAGGCCAACAAAAAGATCGAGAAGCAGCTGCAGA
AGGACAAGCAGGTCTACCGGGCCACGCACCGCCTGCTGCTGCTGGGTG
CTGGAGAATCTGGTAAAAGCACCATTGTGAAGCAGATGAGGATCCTGC
ATGTTAATGGGTTTAATGGAGAGGGCGGCGATGGTGAGAAGGCAACCA
AAGTGCAGGACATCAAAAACAACCTGAAAGAGGCGATTGAAACCATTG
TGGCCGCCATGAGCAACCTGGTGCCCCCGTGAGCTGGCCAACCCCG
AGAACCAGTTCAGAGTGGACTACATTCTGAGTGTGATGAACGTGCCTG
ACTTTGACTTCCCTCCCGAATTCTATGAGCATGCCAAGGCTCTGTGGGA
GGATGAAGGAGTGCGTGCTGCTACGAACGCTCTGGAGGAGGAGGATC
TATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTT
CATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTT
CGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGA
CCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGG
ACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCA
CCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTC

AAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTG
ACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAG
CTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAG
ACCATGGGCTGGGAGGCTCCTCCGAGCGGATGTACCCCGAGGACGGC
GCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGG
CCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGT
GCAGCTGCCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCC
CACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGC
CGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTCTGGAGGAGGA
GGATCTTCCAACGAGTACCAGCTGATTGACTGTGCCAGTACTTCCTGG
ACAAGATCGACGTGATCAAGCAGGCTGACTATGTGCCGAGCGATCAGG
ACCTGCTTCGCTGCCGTGTCCTGACTTCTGGAATCTTTGAGACCAAGTTC
CAGGTGGACAAAGTCAACTTCCACATGTTTGACGTGGGTGGCCAGCGC
GATGAACGCCGCAAGTGGATCCAGTGCTTCAACGATGTGACTGCCATC
ATCTTCGTGGTGGCCAGCAGCAGCTACAACATGGTCATCCGGGAGGAC
AACCAGACCAACCGCCTGCAGGAGGCTCTGAACCTCTTCAAGAGCATC
TGGAACAACAGATGGCTGCGCACCATCTCTGTGATCCTGTTCTCAACA
AGCAAGATCTGCTCGCTGAGAAAGTCCTTGCTGGGAAATCGAAGATTG
AGGACTACTTTCCAGAATTTGCTCGCTACACTACTCCTGAGGATGCTAC
TCCCGAGCCCGGAGAGGACCCACGCGTGACCCGGGCCAAGTACTTCAT
TCGAGATGAGTTTCTGAGGATCAGCACTGCCAGTGGAGATGGGCGTCA
CTACTGCTACCCTCATTTACCTGCGCTGTGGACACTGAGAACATCCGC
CGTGTGTTCAACGACTGCCGTGACATCATTCAGCGCATGCACCTTCGTC
AGTACGAGCTGCTCTAG

GAP43 EGFP Coding Sequence:

ATGCTGTGCTGTATGAGAAGAACCAAACAGGTTGAAAAGAATGATGAG
GACCAAAAGATTGAACAAGATGGTGTCAAGCCGGAAGATAAGGCTCAT
AAGGCTGCGACCAAAATTCAGGCTAGCTTCCGTGGACACATAACAAGG
AAAAAGCTCAAAGGCGAGAAGAAGGGTGATGGTGAGCAAGGGCGAGG
AGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGT
AAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCAC
CTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCC
GTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAAGTGT
TCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGC
CATGCCC GAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGA
CGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCT
GGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAA
CATCCTGGGGCACAAGCTGGAGTACAAC TACAACAGCCACAACGTCTA
TATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGAT
CCGCCACAACATCGAGGACGGCAGCGTGCAAGCTCGCCGACCACTACCA
GCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGACAACCA
CTACCTGAGCACCCAGTCCAAGCTTAGCAAAGACCCCAACGAGAAGCG
CGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCGGGATCACTCTC
GGCATGGACGAGCTGTACAAGTAG

GAP43 mCherry Coding Sequence:

ATGCTGTGCTGTATGAGAAGAACCAAACAGGTTGAAAAGAATGATGAG
GACCAAAAGATTGAACAAGATGGTGTCAAGCCGGAAGATAAGGCTCAT
AAGGCTGCGACCAAAATTCAGGCTAGCTTCCGTGGACACATAACAAGG

AAAAAGCTCAAAGGCGAGAAGAAGGGTGATGGTGAGCAAGGGCGAGG
AGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACA
TGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCG
AGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCA
AGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCAT
GTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTA
CTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAAC
TTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAG
GACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCT
CCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCT
CCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGC
AGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGA
CCACCTACAAGGCCAAGAAGCCCGTGAGCTGCCCCGGCGCCTACAACG
TCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGT
GGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGA
CGAGCTGTACAAGTAG

**GAP43 mCherry-RGSLVPR-EGFP (Positive FRET Control) Coding
Sequence:**

ATGCTGTGCTGTATGAGAAGAACCAAACAGGTTGAAAAGAATGATGAG
GACCAAAAGATTGAACAAGATGGTGTCAAGCCGGAAGATAAGGCTCAT
AAGGCTGCGACCAAAATTCAGGCTAGCTTCCGTGGACACATAACAAGG
AAAAAGCTCAAAGGCGAGAAGAAGGGTGATGGTGAGCAAGGGCGAGG
AGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACA
TGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCG
AGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCA
AGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCAT
GTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTA
CTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAAC

TTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAG
GACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCT
CCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCT
CCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGC
AGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGA
CCACCTACAAGGCCAAGAAGCCCGTGCGAGCTGCCCGGCGCCTACAACG
TCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGT
GGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGA
CGAGCTGTACAAGCGGGGCAGCCTGGTCCCTGGAATGGTGAGCAAGGG
CGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGG
CGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGA
TGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAG
CTGCCCGTGCCCTGGCCCCACCCTCGTGACCACCCTGACCTACGGCGTG
AGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAA
GTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAG
GACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGAC
ACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGAC
GGCAACATCCTGGGGCACAAGCTGGAGTACAACACTACAACAGCCACAAC
GTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTC
AAGATCCGCCACAACATCGAGGACGGCAGCGTGCGAGCTCGCCGACCAC
TACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGAC
AACCCTACCTGAGCACCCAGTCCAAGCTTAGCAAAGACCCCAACGAG
AAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATC
ACTCTCGGCATGGACGAGCTGTACAAGTAG

Gai Minigene Casette Sequence:

GCCGCCACCATGGGAATAAAAAATAATCTAAAGATTGTGGTCTCTTT

Gai Minigene Casette Sequence:

GCCGCCACCATGGGACAGCGCATGCACCTTCGTCAGTACGAGCTGCTC

E. cAMP GLO ASSAY STATISTICAL ANALYSIS

A Unpaired t test		B Unpaired t test			
1	Table Analyzed	Data 2	1	Table Analyzed	Data 2
2			2		
3	Column D	Wild Type Goi Quinpirole	3	Column F	Goi 91 mCherry Quinpirole
4	vs.	vs.	4	vs.	vs.
5	Column C	Wild Type Goi	5	Column E	Goi 91 mCherry
6			6		
7	Unpaired t test		7	Unpaired t test	
8	P value	0.0003	8	P value	0.0347
9	P value summary	***	9	P value summary	*
10	Significantly different (P < 0.05)?	Yes	10	Significantly different (P < 0.05)?	Yes
11	One- or two-tailed P value?	Two-tailed	11	One- or two-tailed P value?	Two-tailed
12	t, df	t=57.76, df=2	12	t, df	t=3.683, df=3
13			13		
14	How big is the difference?		14	How big is the difference?	
15	Mean of column C	93.34	15	Mean of column E	61.87
16	Mean of column D	140.1	16	Mean of column F	96.97
17	Difference between means (D - C) ± SE	46.76 ± 0.8095	17	Difference between means (F - E) ± SE	35.10 ± 9.530
18	95% confidence interval	43.27 to 50.24	18	95% confidence interval	4.771 to 65.43
19	R squared (eta squared)	0.9994	19	R squared (eta squared)	0.8189
20			20		

C Unpaired t test		D Unpaired t test			
1	Table Analyzed	Data 2	1	Table Analyzed	Data 2
2			2		
3	Column H	Goi 121 mCherry Quinpirole	3	Column B	Goi 91 EGFP Quinpirole
4	vs.	vs.	4	vs.	vs.
5	Column G	Goi 121 mCherry	5	Column A	Goi 91 EGFP
6			6		
7	Unpaired t test		7	Unpaired t test	
8	P value	0.4627	8	P value	0.0323
9	P value summary	ns	9	P value summary	*
10	Significantly different (P < 0.05)?	No	10	Significantly different (P < 0.05)?	Yes
11	One- or two-tailed P value?	Two-tailed	11	One- or two-tailed P value?	Two-tailed
12	t, df	t=0.7949, df=5	12	t, df	t=2.772, df=6
13			13		
14	How big is the difference?		14	How big is the difference?	
15	Mean of column G	165.4	15	Mean of column A	54.35
16	Mean of column H	187.3	16	Mean of column B	117.7
17	Difference between means (H - G) ± SE	21.96 ± 27.62	17	Difference between means (B - A) ± SE	63.31 ± 22.84
18	95% confidence interval	-49.05 to 92.96	18	95% confidence interval	7.430 to 119.2
19	R squared (eta squared)	0.1122	19	R squared (eta squared)	0.5616
20			20		

Figure E. 1: Statistical analysis of G α i fusion protein functional assay results. A) Wild type group signal comparison with and without CGS+Quinpirole treatment, B) G α i 91 mCherry fusion protein signal comparison with and without CGS+Quinpirole treatment, C) G α i 121 mCherry fusion protein signal comparison with and without CGS+Quinpirole treatment, D) G α i 91 EGFP fusion protein signal comparison with and without CGS+Quinpirole treatment.

F. STATISTICAL ANALYSIS OF THE CONFOCAL MICROSCOPY RESULTS Of *Gai-Gas* FRET PAIRS

ANOVA results		Multiple comparisons					
Ordinary one-way ANOVA							
Multiple comparisons							
1	Number of families	1					
2	Number of comparisons per family	6					
3	Alpha	0.05					
4							
5	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
6	<i>Gai</i> 121- <i>Gαs</i> 73-85 vs. <i>Gai</i> 91- <i>Gαs</i> 73-85	0.0007713	-0.004557 to 0.006099	No	ns	0.9819	A-B
7	<i>Gai</i> 121- <i>Gαs</i> 73-85 vs. Gap43 EGFP +Gap43 mCherry	-0.007123	-0.01245 to -0.001795	Yes	**	0.0036	A-C
8	<i>Gai</i> 121- <i>Gαs</i> 73-85 vs. Gap43 mCherry-L-EGFP	-0.001780	-0.007108 to 0.003548	No	ns	0.8223	A-D
9	<i>Gai</i> 91- <i>Gαs</i> 73-85 vs. Gap43 EGFP +Gap43 mCherry	-0.007894	-0.01322 to -0.002566	Yes	***	0.0010	B-C
10	<i>Gai</i> 91- <i>Gαs</i> 73-85 vs. Gap43 mCherry-L-EGFP	-0.002551	-0.007879 to 0.002777	No	ns	0.6014	B-D
11	Gap43 EGFP +Gap43 mCherry vs. Gap43 mCherry-L-EGFP	0.005343	1.468e-005 to 0.01067	Yes	*	0.0491	C-D
12							

Figure F. 1: Ordinary One way ANOVA analysis of *Gai*(121) EGFP-*Gαs*(73-85)mCherry , *Gai*(91) EGFP-*Gαs*(73-85)mCherry and Gap43 EGFP+ Gap43 mCherry negative control group confocal microscope results calculated with Pix FRET.

G. MONOCHROMATOR PLATE READER RESULT STATISTICAL ANALYSYS OF G α i-G α s FRET PAIRS

ANOVA results										Multiple comparisons									
Ordinary one-way ANOVA																			
1	Number of families	1																	
2	Number of comparisons per family	6																	
3	Alpha	0.05																	
4																			
5	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value													
6	Goi 121 EGFP-Gus 73-85 mCherry vs. Goi 91 EGFP-Gus 73-85 mCherry	-0.001334	-0.003049 to 0.0003799	No	ns	0.1634				A-B									
7	Goi 121 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP-L-mCherry	0.0001492	-0.001565 to 0.001864	No	ns	0.9947				A-C									
8	Goi 121 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry	0.005923	0.004209 to 0.007637	Yes	****	<0.0001				A-D									
9	Goi 91 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP-L-mCherry	0.001484	-0.0002307 to 0.003198	No	ns	0.1049				B-C									
10	Goi 91 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry	0.007257	0.005543 to 0.008972	Yes	****	<0.0001				B-D									
11	Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43 mCherry	0.005774	0.004059 to 0.007488	Yes	****	<0.0001				C-D									
12																			
13	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF										
14	Goi 121 EGFP-Gus 73-85 mCherry vs. Goi 91 EGFP-Gus 73-85 mCherry	0.01119	0.01252	-0.001334	0.0006125	6	6	3.081	20										
15	Goi 121 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP-L-mCherry	0.01119	0.01104	0.0001492	0.0006125	6	6	0.3446	20										
16	Goi 121 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry	0.01119	0.005295	0.005923	0.0006125	6	6	13.68	20										
17	Goi 91 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP-L-mCherry	0.01252	0.01104	0.001484	0.0006125	6	6	3.426	20										
18	Goi 91 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP + Gap43 mCherry	0.01252	0.005295	0.007257	0.0006125	6	6	16.76	20										
19	Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43 mCherry	0.01104	0.005295	0.005774	0.0006125	6	6	13.33	20										
20																			
21																			
22																			

Figure G. 1: G α i(121) EGFP-G α s(73-85)mCherry , G α i(91) EGFP-G α s(73-85)mCherry and Gap43 FRET control groups mCherry spectral peak region result analysis with Ordinary One way ANOVA .

ANOVA results										Multiple comparisons									
Ordinary one-way ANOVA																			
1	Number of families	1																	
2	Number of comparisons per family	6																	
3	Alpha	0.05																	
4																			
5	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value													
6	Goi 121 EGFP-Gus 73-85 mCherry vs. Goi 91 EGFP-Gus 73-85 mCherry	-0.04260	-0.09539 to 0.01020	No	ns	0.1485				A-B									
7	Goi 121 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP-L-mCherry	0.04106	0.01128 to 0.07084	Yes	**	0.0039				A-C									
8	Goi 121 EGFP-Gus 73-85 mCherry vs. gap43 EGFP + gap43mCherry	0.2507	0.2209 to 0.2804	Yes	****	<0.0001				A-D									
9	Goi 91 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP-L-mCherry	0.08366	0.03208 to 0.1352	Yes	***	0.0007				B-C									
10	Goi 91 EGFP-Gus 73-85 mCherry vs. gap43 EGFP + gap43mCherry	0.2933	0.2417 to 0.3448	Yes	****	<0.0001				B-D									
11	Gap43 EGFP-L-mCherry vs. gap43 EGFP + gap43mCherry	0.2096	0.1820 to 0.2372	Yes	****	<0.0001				C-D									
12																			
13	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF										
14	Goi 121 EGFP-Gus 73-85 mCherry vs. Goi 91 EGFP-Gus 73-85 mCherry	0.4099	0.4525	-0.04260	0.01945	9	2	3.097	31										
15	Goi 121 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP-L-mCherry	0.4099	0.3688	0.04106	0.01097	9	12	5.293	31										
16	Goi 121 EGFP-Gus 73-85 mCherry vs. gap43 EGFP + gap43mCherry	0.4099	0.1592	0.2507	0.01097	9	12	32.31	31										
17	Goi 91 EGFP-Gus 73-85 mCherry vs. Gap43 EGFP-L-mCherry	0.4525	0.3688	0.08366	0.01900	2	12	6.226	31										
18	Goi 91 EGFP-Gus 73-85 mCherry vs. gap43 EGFP + gap43mCherry	0.4525	0.1592	0.2933	0.01900	2	12	21.82	31										
19	Gap43 EGFP-L-mCherry vs. gap43 EGFP + gap43mCherry	0.3688	0.1592	0.2096	0.01016	12	12	29.18	31										

Figure G. 2: G α i(121) EGFP-G α s(73-85)mCherry , G α i(91) EGFP-G α s(73-85)mCherry and Gap43 FRET control groups Acceptor/Donor peak area result analysis with Ordinary One way ANOVA .

H. STATISTICAL ANALYSIS OF THE CONFOCAL MICROSCOPY RESULTS Of *Gai*-*Gas* FRET PAIRS WITH *Gai* and *Gas* PROTEIN SPECIFIC MINIGENES

Ordinary one-way ANOVA							
Multiple comparisons							
1	Number of families	1					
2	Number of comparisons per family	3					
3	Alpha	0.05					
4							
5	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
6	<i>Gai</i> 91- <i>Gas</i> 73-85 vs. Column B	0.004886	0.004120 to 0.005652	Yes	****	<0.0001	A-B
7	<i>Gai</i> 91- <i>Gas</i> 73-85 vs. <i>Gai</i> 121- <i>Gas</i> 73-85	0.006141	0.005376 to 0.006907	Yes	****	<0.0001	A-C
8	Column B vs. <i>Gai</i> 121- <i>Gas</i> 73-85	0.001256	0.0004897 to 0.002021	Yes	***	0.0005	B-C
9							
10	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2
11	<i>Gai</i> 91- <i>Gas</i> 73-85 vs. Column B	0.008664	0.003778	0.004886	0.0003233	48	48
12	<i>Gai</i> 91- <i>Gas</i> 73-85 vs. <i>Gai</i> 121- <i>Gas</i> 73-85	0.008664	0.002523	0.006141	0.0003233	48	48
13	Column B vs. <i>Gai</i> 121- <i>Gas</i> 73-85	0.003778	0.002523	0.001256	0.0003233	48	48
14							
15							
16							

Figure H. 1: LSM Confocal microscope result from *Gai*(121) EGFP-*Gas*(73-85)mCherry , *Gai*(91) EGFP-*Gas*(73-85)mCherry and *Gai*(121) EGFP-*Gas*(73-85)mCherry with minigene FRET groups analyzed with Ordinary One Way Anova analysis.

I. MONOCHROMATOR PLATE READER RESULT STATISTICAL ANALYSYS OF *Gai*-*Gas* FRET PAIRS WITH *Gai* and *Gas* PROTEIN SPECIFIC MINIGENES

ANOVA results										Multiple comparisons									
Ordinary one-way ANOVA										Multiple comparisons									
1	Number of families	1																	
2	Number of comparisons per family	6																	
3	Alpha	0.05																	
4																			
5	Tukey's multiple comparisons test		Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value												
6	Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43 mCherry		0.005774	0.004339 to 0.007208	Yes	****	<0.0001	A-B											
7	Gap43 EGFP-L-mCherry vs. Gai 121 EGFP-Gas 73-85 mCherry		-0.0001492	-0.001584 to 0.001285	No	ns	0.9911	A-C											
8	Gap43 EGFP-L-mCherry vs. Column D		-0.0006126	-0.002047 to 0.0008221	No	ns	0.6368	A-D											
9	Gap43 EGFP + Gap43 mCherry vs. Gai 121 EGFP-Gas 73-85 mCherry		-0.005923	-0.007358 to -0.004488	Yes	****	<0.0001	B-C											
10	Gap43 EGFP + Gap43 mCherry vs. Column D		-0.006386	-0.007821 to -0.004952	Yes	****	<0.0001	B-D											
11	Gai 121 EGFP-Gas 73-85 mCherry vs. Column D		-0.004634	-0.001958 to 0.0009713	No	ns	0.8029	C-D											
12																			
13	Test details		Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF									
14	Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43 mCherry		0.01104	0.005265	0.005774	0.0005126	6	6	15.93	20									
15	Gap43 EGFP-L-mCherry vs. Gai 121 EGFP-Gas 73-85 mCherry		0.01104	0.01119	-0.0001492	0.0005126	6	6	0.4118	20									
16	Gap43 EGFP-L-mCherry vs. Column D		0.01104	0.01165	-0.0006126	0.0005126	6	6	1.690	20									
17	Gap43 EGFP + Gap43 mCherry vs. Gai 121 EGFP-Gas 73-85 mCherry		0.005265	0.01119	-0.005923	0.0005126	6	6	16.34	20									
18	Gap43 EGFP + Gap43 mCherry vs. Column D		0.005265	0.01165	-0.006386	0.0005126	6	6	17.62	20									
19	Gai 121 EGFP-Gas 73-85 mCherry vs. Column D		0.01119	0.01165	-0.004634	0.0005126	6	6	1.278	20									
20																			
21																			
22																			
23																			
24																			
25																			
26																			

Figure I. 1: Monochromator plate reader results of *Gai*(121) EGFP-*Gas*(73-85)mCherry , *Gai*(91) EGFP-*Gas*(73-85)mCherry and *Gai*(121) EGFP-*Gas*(73-85)mCherry with minigene FRET groups Ordinary One Way Anova analysis

ANOVA results										Multiple comparisons									
Ordinary one-way ANOVA										Multiple comparisons									
1	Number of families	1																	
2	Number of comparisons per family	6																	
3	Alpha	0.05																	
4																			
5	Tukey's multiple comparisons test		Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value												
6	Gai 121 EGFP-Gas 73-85 mCherry vs. Column B		-0.02257	-0.04319 to -0.001957	Yes	*	0.0303	A-B											
7	Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP-L-mCherry		0.01344	-0.01064 to 0.03751	No	ns	0.3925	A-C											
8	Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43mCherry		0.1793	0.1552 to 0.2034	Yes	****	<0.0001	A-D											
9	Column B vs. Gap43 EGFP-L-mCherry		0.03601	0.01115 to 0.06087	Yes	**	0.0046	B-C											
10	Column B vs. Gap43 EGFP + Gap43mCherry		0.2019	0.1770 to 0.2267	Yes	****	<0.0001	B-D											
11	Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43mCherry		0.1659	0.1381 to 0.1937	Yes	****	<0.0001	C-D											
12																			
13	Test details		Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF									
14	Gai 121 EGFP-Gas 73-85 mCherry vs. Column B		0.3022	0.3248	-0.02257	0.007024	6	5	4.545	13									
15	Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP-L-mCherry		0.3022	0.2888	0.01344	0.008202	6	3	2.317	13									
16	Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43mCherry		0.3022	0.1229	0.1793	0.008202	6	3	30.91	13									
17	Column B vs. Gap43 EGFP-L-mCherry		0.3248	0.2888	0.03601	0.008471	5	3	6.012	13									
18	Column B vs. Gap43 EGFP + Gap43mCherry		0.3248	0.1229	0.2019	0.008471	5	3	33.70	13									
19	Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43mCherry		0.2888	0.1229	0.1659	0.009471	3	3	24.77	13									
20																			

Figure I. 2: Acceptor/Donor spectral peak area results of *Gai*(121) EGFP-*Gas*(73-85)mCherry , *Gai*(91) EGFP-*Gas*(73-85)mCherry and *Gai*(121) EGFP-*Gas*(73-85)mCherry with minigene FRET groups Ordinary One Way Anova analysis

J. WITH/WITHOUT CGS+QUINPIROLE TREATMENT RESULT ANALYSIS OF Gai-Gas FRET GROUP WITH/WITHOUT MINIGENE

Ordinary one-way ANOVA							
Multiple comparisons							
1	Number of families	1					
2	Number of comparisons per family	15					
3	Alpha	0.05					
4							
5	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
6	Gai 121 EGFP-Gas 73-85 mCherry vs. Column B	-0.0002228	-0.002092 to 0.001646	No	ns	0.9991	A-B
7	Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 mCherry-LEGFP	0.0001492	-0.001720 to 0.002018	No	ns	0.9999	A-C
8	Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43mCherry	0.0005923	0.004054 to 0.007792	Yes	****	<0.0001	A-D
9	Gai 121 EGFP-Gas 73-85 mCherry vs. Column E	-0.0007222	-0.002591 to 0.001147	No	ns	0.8449	A-E
10	Gai 121 EGFP-Gas 73-85 mCherry vs. Column F	0.0005139	-0.001355 to 0.002383	No	ns	0.9582	A-F
11	Column B vs. Gap43 mCherry-LEGFP	0.0003721	-0.001497 to 0.002241	No	ns	0.9898	B-C
12	Column B vs. Gap43 EGFP + Gap43mCherry	0.006146	0.004277 to 0.008015	Yes	****	<0.0001	B-D
13	Column B vs. Column E	-0.0004994	-0.002368 to 0.001370	No	ns	0.9629	B-E
14	Column B vs. Column F	0.0007367	-0.001132 to 0.002606	No	ns	0.8338	B-F
15	Gap43 mCherry-LEGFP vs. Gap43 EGFP + Gap43mCherry	0.005774	0.003905 to 0.007642	Yes	****	<0.0001	C-D
16	Gap43 mCherry-LEGFP vs. Column E	-0.0008714	-0.002740 to 0.0009975	No	ns	0.7160	C-E
17	Gap43 mCherry-LEGFP vs. Column F	0.0003646	-0.001504 to 0.002234	No	ns	0.9907	C-F
18	Gap43 EGFP + Gap43mCherry vs. Column E	-0.006645	-0.008514 to -0.004776	Yes	****	<0.0001	D-E
19	Gap43 EGFP + Gap43mCherry vs. Column F	-0.005409	-0.007278 to -0.003540	Yes	****	<0.0001	D-F
20	Column E vs. Column F	0.001236	-0.0006328 to 0.003105	No	ns	0.3597	E-F

Figure J. 1: Ordinary One Way ANOVA analysis of mCherry spectrum peak normalized area comparison of Gai-Gas FRET pairs with/without Gα protein specific minigenes and with/without CGS+Quinpirole treatment.

ANOVA results							
Multiple comparisons							
1	Number of families	1					
2	Number of comparisons per family	15					
3	Alpha	0.05					
4							
5	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
6	Gai 121 EGFP-Gas 73-85 mCherry vs. Column B	-0.02535	-0.04706 to -0.003635	Yes	*	0.0179	A-B
7	Gai 121 EGFP-Gas 73-85 mCherry vs. Column C	-0.05088	-0.07259 to -0.02917	Yes	****	<0.0001	A-C
8	Gai 121 EGFP-Gas 73-85 mCherry vs. Column D	-0.02802	-0.05147 to -0.004566	Yes	*	0.0151	A-D
9	Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP-L-mCherry	0.007202	-0.01625 to 0.03066	No	ns	0.9116	A-E
10	Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43mCherry	-0.1731	0.1496 to 0.1965	Yes	****	<0.0001	A-F
11	Column B vs. Column C	-0.02553	-0.04725 to -0.003818	Yes	*	0.0170	B-C
12	Column B vs. Column D	-0.002670	-0.02612 to 0.02078	No	ns	0.9989	B-D
13	Column B vs. Gap43 EGFP-L-mCherry	0.03255	0.009097 to 0.05600	Yes	**	0.0046	B-E
14	Column B vs. Gap43 EGFP + Gap43mCherry	0.1984	0.1750 to 0.2219	Yes	****	<0.0001	B-F
15	Column C vs. Column D	0.02286	-0.0005920 to 0.04631	No	ns	0.0581	C-D
16	Column C vs. Gap43 EGFP-L-mCherry	0.05808	0.03463 to 0.08154	Yes	****	<0.0001	C-E
17	Column C vs. Gap43 EGFP + Gap43mCherry	0.2239	0.2005 to 0.2474	Yes	****	<0.0001	C-F
18	Column D vs. Gap43 EGFP-L-mCherry	0.03522	0.01015 to 0.06029	Yes	**	0.0041	D-E
19	Column D vs. Gap43 EGFP + Gap43mCherry	0.2011	0.1760 to 0.2261	Yes	****	<0.0001	D-F
20	Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43mCherry	0.1659	0.1408 to 0.1909	Yes	****	<0.0001	E-F

Figure J. 2: One way ANOVA analysis of Acceptor/Donor spectral area normalized peak results of Gai-Gas FRET pairs with/without Gα protein specific minigenes and with/without CGS+Quinpirole treatment.

K. CURVE FITTING STATISTICAL ANALYSIS

ANOVA results		X		Multiple	
2way ANOVA Multiple comparisons					
Within each row, compare columns (simple effects within rows)					
Number of families		3			
Number of comparisons per family		6			
Alpha		0.05			
Tukey's multiple comparisons test		Mean Diff.	95.00% CI of diff.	Significant?	Summary
negative					Adjusted P Value
Gai 91 EGFP-Gas 73-85 mCherry vs. Gai 121 EGFP-Gas 73-85 mCherry		-197.4	-463.5 to 68.77	No	ns
Gai 91 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP-L-mCherry		60.37	-205.8 to 326.5	No	ns
Gai 91 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43mCherry		-1116	-1386 to -846.8	Yes	****
Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP-L-mCherry		257.7	-8.396 to 523.9	No	ns
Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43mCherry		-919.0	-1189 to -649.4	Yes	****
Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43mCherry		-1177	-1446 to -907.2	Yes	****
membrane					
Gai 91 EGFP-Gas 73-85 mCherry vs. Gai 121 EGFP-Gas 73-85 mCherry		-154.5	-420.6 to 111.6	No	ns
Gai 91 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP-L-mCherry		-489.6	-755.7 to -223.5	Yes	****
Gai 91 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43mCherry		137.9	-131.7 to 407.5	No	ns
Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP-L-mCherry		-335.1	-601.2 to -68.98	Yes	**
Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43mCherry		292.4	22.80 to 562.0	Yes	*
Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43mCherry		627.5	357.9 to 897.1	Yes	****
organelle					
Gai 91 EGFP-Gas 73-85 mCherry vs. Gai 121 EGFP-Gas 73-85 mCherry		92.92	-173.2 to 359.0	No	ns
Gai 91 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP-L-mCherry		71.46	-194.7 to 337.6	No	ns
Gai 91 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43mCherry		221.8	-47.76 to 491.4	No	ns
Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP-L-mCherry		-21.46	-287.6 to 244.7	No	ns
Gai 121 EGFP-Gas 73-85 mCherry vs. Gap43 EGFP + Gap43mCherry		128.9	-140.7 to 398.5	No	ns
Gap43 EGFP-L-mCherry vs. Gap43 EGFP + Gap43mCherry		150.4	-119.2 to 420.0	No	ns

Figure K. 1: Signal localization curve fit Two way ANOVA analysis results of FRET pairs.

Ordinary one-way ANOVA								
Multiple comparisons								
1	Number of families	1						
2	Number of comparisons per family	6						
3	Alpha	0.05						
4								
5	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value		
6	1 vs. 2	-154.5	-362.5 to 53.51	No	ns	0.2159	A-B	
7	1 vs. 3	-489.6	-697.6 to -281.6	Yes	****	<0.0001	A-C	
8	1 vs. 4	138.7	-69.28 to 346.7	No	ns	0.3046	A-D	
9	2 vs. 3	-335.1	-543.1 to -127.1	Yes	***	0.0004	B-C	
10	2 vs. 4	293.2	85.20 to 501.2	Yes	**	0.0022	B-D	
11	3 vs. 4	628.3	420.3 to 836.3	Yes	****	<0.0001	C-D	
12								
13	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q
14	1 vs. 2	156.3	310.7	-154.5	79.18	20	20	2.759
15	1 vs. 3	156.3	645.8	-489.6	79.18	20	20	8.744
16	1 vs. 4	156.3	17.55	138.7	79.18	20	20	2.478
17	2 vs. 3	310.7	645.8	-335.1	79.18	20	20	5.985
18	2 vs. 4	310.7	17.55	293.2	79.18	20	20	5.237
19	3 vs. 4	645.8	17.55	628.3	79.18	20	20	11.22
20								
21								

Figure K. 2: Membrane curve fit result One Way ANOVA analysis of FRET pairs