IN VIVO DETECTION OF YEAST ALPHA MATING PHEROMONE RECEPTOR STE2P HOMODIMERIZATION BY FRET

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ABSTRACT

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Ste2p is an alpha type pheromone sensing receptor of 'a' type *Saccharomyces cerevisiae* cells. Yeast life cycle could be haploid or diploid due to the signal sensed by Ste2p. This receptor is a G protein coupled receptor (GPCR). GPCRs are one of the most important drug targets because they are playing key roles in cell signaling. They have seven transmembrane domains and linked with a G protein in the cytosol.

FRET is a method that is used for detecting protein-protein interactions by using the resonance energy transfer between fluorophores. The fluorophores are named as donor and acceptor. When two fluorophores are in close proximity to each other FRET signal is generated and this signal can be quantified to determine the distance between the donor and the acceptor. For this purpose Ste2p is labeled with two different fluorophores EGFP and mCherry, green and red, respectively. During the study two different positions of receptor was labeled with the two fluorophores. Yeast cells were transfected with these vectors bearing the fusion gene of receptor and fluorophores. After transfection of the two labeled receptors, the presence of FRET signal was observed under confocal microscope.

FRET provides chance of observing live cells without causing harm. In this study in addition to detection of homodimerization we studied effect of agonist on receptor dimerization and fluorescent protein position on FRET efficiency.

Keywords: FRET, Ste2p, yeast, *Saccharomyces cerevisiae*, homodimerization, GPCR, *in vivo*

ÖZ

MAYA ALFA FEROMON RESEPTÖRÜ STE2P'NİN HOMODİMERİZASYONUNUN FRET İLE *IN VIVO* GÖSTERİLMESİ

Bulut, Giray

Yüksek Lisans. Biyoloji Bölümü

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Ste2p a tipi *Saccharomyces cerevisiae* hücrelerinde bulunan ve alfa tipi feromon algılayan bir reseptördür. Maya hücrelerinin yaşam döngüsü Ste2p tarafından algılanan sinyale göre haploid ya da diploid olabilir. Bu reseptör bir tür G-proteine kenetli reseptördür (GPKR). GPKRler sinyal iletimindeki önemli görevlerinden dolayı en önemli ilaç hedeflerinden biridir. Bu reseptörler yedi transmembran bölümden oluşur ve hücrenin içerisinde G-proteine bağlanırlar.

FRET Floroforlar arasındaki rezonans enerji transferi ile proteinler arası etkileşimleri gösterebilen bir metottur. Bu floroforlar donör ve akseptör olarak adlandırılır. İki florofor birbirine yeterince yaklaştığında FRET sinyali gözlenir ve bu sinyal akseptör ile donör arasındaki uzaklığı hesaplamak için kullanılabilir. Bu amaçla Ste2p iki farklı florofor olan EGFP ve mCherry ile işaretlenmiştir, bunlar sırasıyla yeşil ve kırmızı renktedir. Çalışma süresince reseptörün iki farklı aminoasit pozisyonu bu iki floroforla gen düzeyinde işaretlenmiştir. Maya hücreleri bu işaretli reseptörü

taşıyan plazmidlerle transfekte edilmiştir. Transfeksiyondan sonra aynı hücredeki farklı renkle işaretli proteinlerden konfokal mikroskop ile FRET sinyali alınmıştır.

FRET canlı hücrelere zarar vermeden inceleme olanağı sağlamaktadır. Bu çalışmada homodimerizasyona ek olarak, feromonun ve florofor pozisyonunun da FRET üzerindeki etkileri çalışılmıştır.

Anahtar kelimeler: FRET, maya, *Saccharomyces cerevisae*, homodimerizasyon, GPKR, *in vivo*

To My Sister and Family,

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LIST OF SYMBOLS AND ABBREVIATIONS

AFM	Atomic force microscopy
Bar	Barrier (to α-factor diffusion)
Bem	Bud emergence
Bp	Base pair
BRET	Bioluminescence Resonance Energy Transfer
cAMP	cyclic AMP
Cdc	Cell division control
DLS	dynamic light scattering
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced Green Florescent Protein
EL	Extracellular lopp
ER	Endoplasmic Reticulum
Far	Factor arrest
FRET	Fluorescence/Förster Resonance Energy Transfer
GDP	Guanosine Diphosphate
GFP	Green Fluorescence Protein
Gi	Inhibitory Gα subunit
Golf	Olfactory Gα subunit
Gpa1	G-protein alpha subunit
Gs	Stimulatory Gα subunit
GTP	Guanosine Triphosphate
IL	Intracellular loop
kb	Kilobase
Kss1	Kinase-supressor of Sst2
LB	Luria Bertani
MAP	Mitogen Activated Protein
MLT	Media lack of Tryptophane
MLTU	Media lack of Tryptophane and Uracil

MLU	Media lack of Uracil
Msg5	Multicopy suppressor of *GPA1* deletion
PAK	p21-activated protein kinase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RE	Restriction Enzyme
Rpm	Revolution per Minute
Ste	Sterile
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TM	Transmembrane
UV	Ultraviolet
YEPD	Yeast extract-peptone-dextrose
rpm	Revolutions per minute
RNA	Ribonucleic acid

CHAPTER1

1. INTRODUCTION

1.1 G Protein-coupled receptors

G protein-coupled receptors (GPCRs) are the largest class of cell surface receptors. Human genome contains nearly 1000 genes for G-protein coupled receptors (GPCRs) (Takeda, Kadowaki, Haga, Takaesu, & Mitaku, 2002). Their general structure composed of α-helical, hydrophobic seven transmembrane (TM) domains with extracellular N terminus and intracellular C terminus (Figure 1.1) (Kobilka, 2007). Transmembrane (TM) domains are more conserved in GPCRs compared to extracellular and intracellular regions. As a common property, these receptors couple with a heterotrimeric G protein at its cytosolic loop.

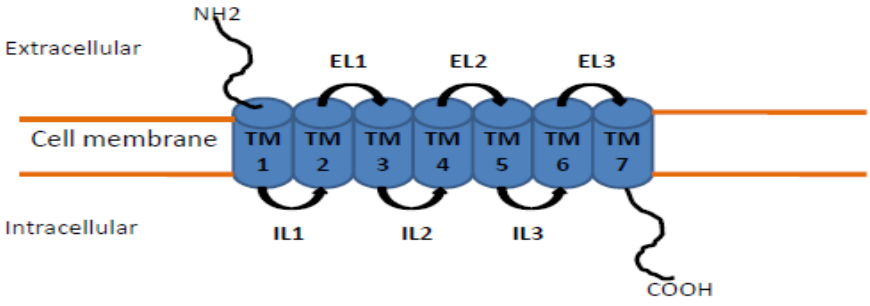

Figure 1.1. Common structure of GPCRs. Seven trans membrane domains (TMs), intracellular C tail and extracellular N tail.

GPCR super family can be divided into five main families according to their sequence similarities and functions. These families are named as; adhesion, taste, glutamate, rhodopsin and secretin receptors (Fredriksson, Lagerström, Lundin & Schiöth, 2003).

Because of their roles in immune responses, cell growth, signal transduction and sensing environment with various ligands; GPCR superfamily has a very important role in many biological functions and they are explored in many studies in order to understand their mechanism of action. The ligands that bind and activate GPCRs can be extracellular chemicals; painkiller drugs, neurotransmitters, chemoattractants, ions and also hormones or, these receptors can sense, taste molecules, odorant molecules and light (Gurevich & Gurevich, 2008). Due to their wide range of ligand repertoire, GPCRs became target of nearly half of the drugs in the market and still one of the main focuses of drug research (Gonzalez-Maeso, 2011). Mutations or malfunctions of GPCRs are related with various diseases such as diabetes, hypertension, cholesterol, obesity and psychotic disorders (Vilardaga, Agnati, Fuxe, & Clruela, 2010). 25% of most selling 100 drugs target GPCRs. Thus these receptors are thought to be one of the most important receptors for drug target and drug design (Veulens & Rodríguez, 2009). List of drugs targeting GPCRs are given in Appendix E.

When ligands bind to GPCRs, they are activated and transfer signal to heterotrimeric G-proteins. G-proteins are composed of α, β and γ subunits and following ligand binding, G-protein binds GTP and undergoes a conformational change on α subunit but not on other subunits (Lambright *et al.*, 1996). After activation, released α subunit with GTP, as well as the βγ subunits interact with various effector molecules in the cell (Conklin *et al.*, 1993, Neer, 1995). Figure 1.2 shows the general GPCR activation mechanisms.

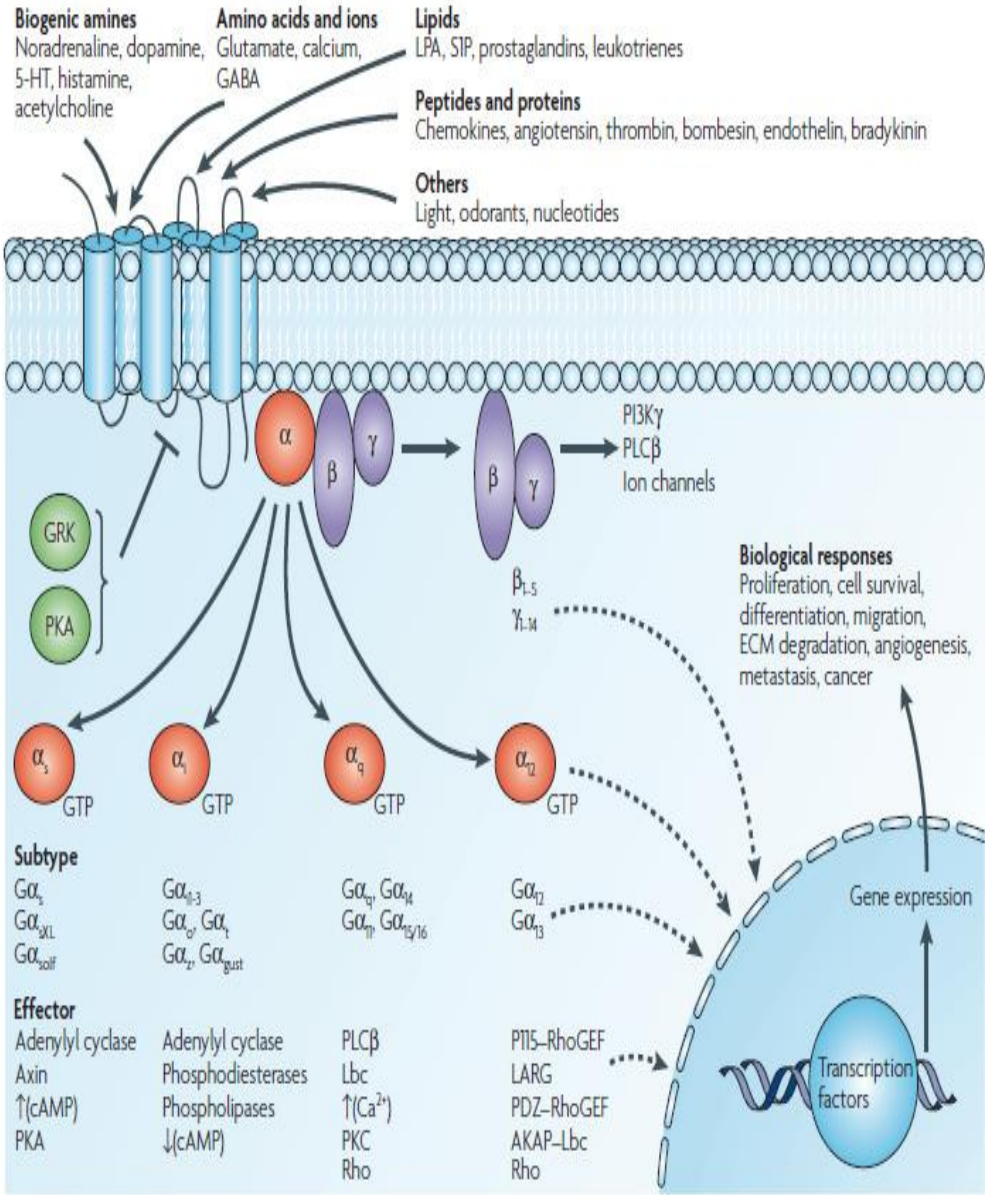

Figure 1.2. GPCR signaling through various ranges of ligands and different pathways according to ligands (Dorsam & Gutkind, 2007).

1.2 GPCRs in yeast and Ste2p

In human genome there are many genes coding for GPCRs. Similarly there are many genes coding for G proteins and regulators of G proteins which are composed of 14 Gα proteins, 5 Gβ, 7 Gγ and 31 regulator of G protein signaling proteins (RGSs). In contrast, in yeast, only two GPCR systems are identified; one for mating, and the other for glucose sensing and only three GPCRs are responsible for these processes: Ste2p, Ste3p and Gpr1p (Thevelein, Lemaire, Versele, 2001).

There are two types of haploid yeast cells; one is type ***a*** (genotype *MAT*a) other is type **α** (genotype *MAT*α). α type cells produce α-factor pheromone which is a 13 amino acid long peptide, its sequence is WHWLQLKPGQPMY and these cells contain Ste3p (a-factor receptor) on the cell membrane. ***a*** type cells produce a-factor pheromone which is a 12 amino acid long peptide with sequence of YIIKGVFWDPAC also covalently attached to farnesyl group and these cells contain Ste2p (α-factor receptor) on the surface (Bardwell, 2004), (Thevelein, Lemaire, Versele, 2001). Both receptors are coupled with Gpa1p which is a Gα protein and Ste4p and Ste18p which are β and γ subunits of G-protein, respectively.

1.3 Pheromone response pathway of yeast

After stimulation of yeast cell with pheromone secreted from nearby opposite cell type, various physiological changes occur in the cell for preparing it to mate. These changes include changes in the expression of 200 genes which constitutes approximately 3% of the yeast genome. Cell cycle arrests at G1 phase and cells extending projections to each other. When cells make projections to each other their cell shape changes and this form is called shmoo cells (Thevelein, Lemaire, Oversee, 2001). Following

the initial changes cell membranes of the two cells fuse and finally their nuclei also fuse.

All these processes take about 4 hours under normal conditions. Yeast cells show similar physiological responses when purified pheromone of opposite mating type is added to the environment. Purification of a-Factor pheromone is difficult and synthesizing it is troublesome. Moreover, it sticks to many surfaces, as a result of that many researchers prefer using Ste2p and synthesized α-Factor pheromone and *MAT* a cells. Changes induced by pheromone addition are not irreversible, and if cells cannot mate for a period of time they turn back to their previous state and continue vegetative growth as haploid cells. Signal transduction pathway of sensing and responding to extracellular pheromone is called as 'yeast mating pheromone response pathway' (Bardwell, 2004).

Pheromone response pathway of *S. cerevisiae* is a well-defined GPCR system with all its components and targets studied in many laboratories. Studies about this pathway were initiated with the isolation of sterile mutants (Hartwell, 1980). Sterile mutants of yeast (STE mutants) cannot mate and these cells will not enter cell cycle arrest and they do not change their shape in response to purified pheromone (Bardwell, 2004).

Also many of the GPCR systems in mammals are similar to this pathway (Pausch, 1997). Thus, ''yeast pheromone pathway'' can be a good model system for understanding GPCR systems, and ligand GPCR interactions. *In vivo* studies are another advantage of yeast system. Moreover, fast growth, fully sequenced genome, simplicity of genetic manipulation and low maintenance cost are other advantages of yeast system (Lee *et al.*, 2007).

These advantages and its suitability for expressing recombinant proteins make yeast a widely used model organism for many studies (Xue, Hsueh, & Heitman, 2008).

Mating process starts with pheromone binding to GPCR found on the cell surface. In figure 1.3, cartoon representation of processes after pheromone binding is shown. This event is similar for all GPCRs found in eukaryotes. and this binding stimulates G-protein that coupled to receptor so GDP to GTP exchange occurs and GTP binds to Gα, triggering Gβγ subunits release from the heterotrimeric G-protein (Bardwell, 2004). Gα might have additional roles on mating process more than regulating the Gβγ (Guo *et al*., 2003; Metodiev, Matheos, Rose, & Stone, 2002). Moreover Gα could remain loosely bound to Gβγ and receptor simultaneously so that Gα maintains regulating Gβγ (Klein, Reuveni, & Levitzki, 2000). The information goes from Gβγ subunits to protein kinase cascade then reaches to targets and nuclear transcription factors (Bardwell, 2004).

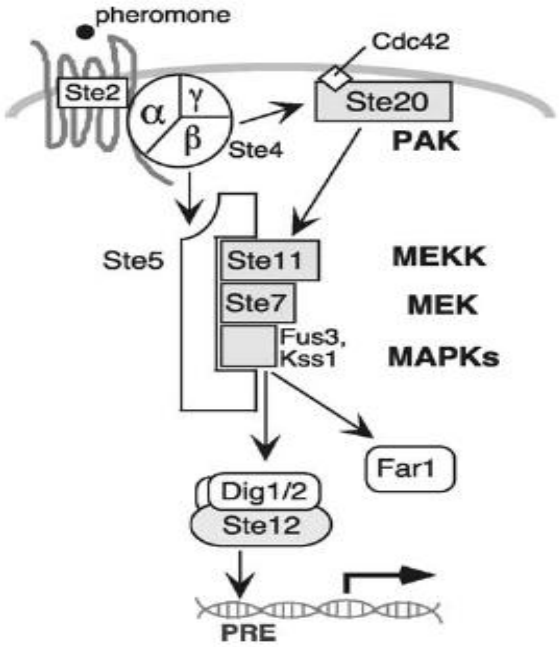

Figure 1.3. Cartoon representation of some elements in yeast mating pathway (Bardwell, 2004).

To sum up, initial pheromone binding to the receptor triggers kinase cascade as a result of which necessary genes for mating are expressed, then cell cycle arrest occurs at G1 phase. After that, cell makes projection towards the highest pheromone concentration site where the mating partner is, finally cells are fused and form diploid form (Bardwell, 2004).

1.4 GPCR oligomerization

Many of the cell surface receptors function as dimers. These dimers could be formed in two ways; first is homodimers which means dimerization occurs between same kind of proteins and other is heterodimerization which occurs between two different proteins. Dimerization could initiate new properties to both receptors different from their monomeric state such as; increased ligand specificity, altered trafficking, mobility and internalization properties (Lohse, 2010).

For many years GPCRs were thought to exist and function as monomers. In literature there are many studies showing GPCRs can function as monomers but these studies do not prove that GPCRs always remain as monomers. On the contrary recent studies show that GPCRs function as dimers or higher oligomers (Angers *et al*., 2002; Lee, O'Dowd, George, 2003; Milligan, *et al*., 2003).

Several methods used in protein-protein interaction were also used for dimerization studies of GPCRs. One of these methods is Co-immunoprecipitation. In Sf9 cell line β2 adrenergic receptors tagged with c-myc- and HA- antibodies were shown to undergo dimerization by using this approach (Hebert *et al*., 1996). Advances in RET (resonance energy transfer) techniques like FRET (fluorescence resonance energy transfer) and BRET (bioluminescence resonance energy transfer) provide opportunity of

studying interaction in living cells. FRET method is applied to studies of Ste2p homodimerization previously. However in previous studies receptors were truncated which are endocytosis mutants (Overton & Blumer, 2000). Ste2p is a widely used model for GPCR studies because of its similarity with other GPCRs (Figure 1.4). At its C-terminal it has sites for phosphorylation and ubiquitination and these sites play role on receptor's desensitization and internalization. Ste2p also coupled with G-protein and stimulates signal to downstream by IC3 (intracellular loop 3) and extracellular loop 2 and 3 (EC2, EC3) and 11 sites at N-terminal are important for α-factor recognition (Jones Jr. & Bennett, 2011).

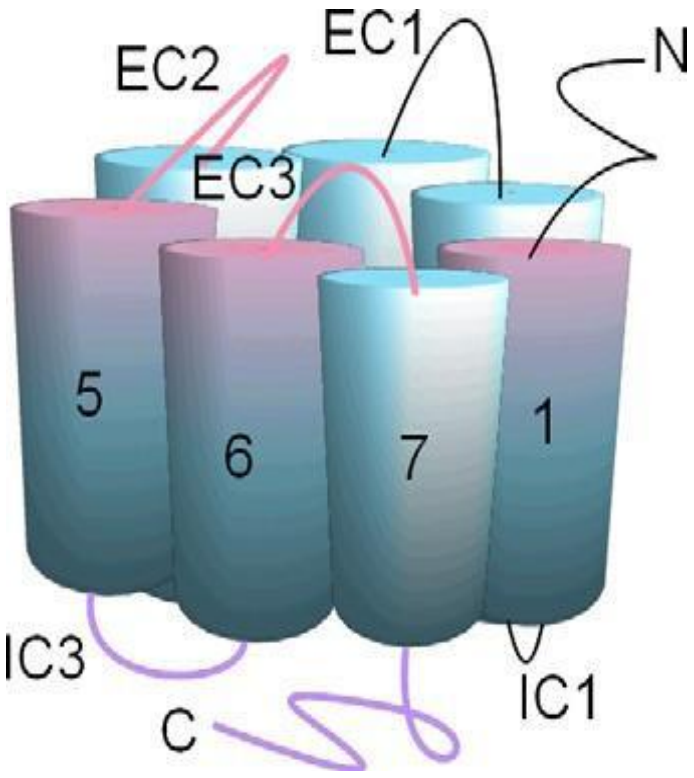

Figure 1.4. Model structure of Ste2 pheromone receptor in *Saccharomyces cerevisiae* (Jones Jr. & Bennett, 2011).

Ste2p homodimerization has been studied with several protein-protein interaction methods. At first, cytoplasmic domain of carboxyl-terminus is deleted which contains DAKSS sequence and it is responsible for endocytosis. As a result of this, internalization is blocked but when mutants expressed with wild type receptors, internalization property is regained, indicating dimerization. This result also supported with biochemical experiments like co-immunoprecipitation (Yesilaltay & Jenness, 2000). In 2000 dimerization is shown in intact cells by using FRET method. According to the results of that study, agonist and antagonist addition does not affect the dimerization of receptor and only dimers of the Ste2p is internalized (Overton & Blumer, 2000). BRET method was used in another study and results are in agreement with Overton and Blumer's results. According to BRET used study for functionality of the receptor, both of the receptors should be functional otherwise, depending on the type of mutation, they lose their functionality partially or completely (Gehret, Bajaj, Naider, & Dumont, 2006).

One of the recent studies about dimerization and ligand relation was conducted using atomic force microscopy (AFM), dynamic light scattering (DLS) and chemical cross linking, *in vitro*. Dimers were obtained in both cases (with pheromone and without pheromone). However, pheromone addition generates more dominant dimers. According to these results, dimer formation is not dependent on pheromone existence in the environment but presence of pheromone improves dimer stability (Shi, *et al.*, 2009).

Several specific contact sites for homodimerization are also defined; TM1 of the Ste2p is one of them with help of TM2 and N-terminal domain. In TM1 GXXXG motif is thought to be crucial because amino acid replacement in this motif prevent homodimer formation (Overton & Blumer, 2002).

1.5 FRET: Powerful tool for *in vivo* detection of protein-protein interaction

FRET stands for Förster's or fluorescence resonance energy transfer. FRET needs two chromophores; one is donor and the other is acceptor. Energy transfer in FRET occurs by non-radiative energy transfer from excited donor to acceptor and this result with emission of acceptor (Förster, 1946). FRET needs several conditions to occur, first of them is spectral overlap, which means emission wavelength of donor chromophore have to overlap with absorption spectrum of the acceptor chromophore. Second condition is the distance between the chromophores for FRET is between 10-100 Å (Milligan, 2004). FRET efficiency is inversely related to the 6^{th} power of the distance between the donor and the acceptor. According to this, even a small distance change can result in significant change in FRET efficiency (Milligan, 2004).

Effect of distance on FRET efficiency is calculated with the following formula; $k_T = (1/ \tau_D) \times (R_0/R)^6$ (Förster, 1946) where, k_T is dipole-dipole transfer rate, τ_D is donor's lifetime fluorescence, R distance of acceptor and donor, R_0 is the Förster distance where half of the maximum FRET efficiency is obtained for selected FRET couple. Figure 1.5 is the cartoon representation of FRET.

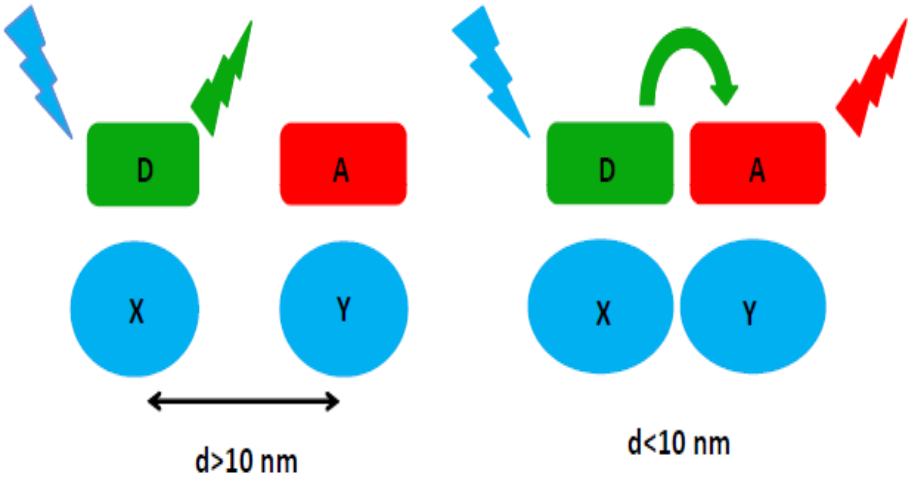

Figure 1.5. Cartoon representation of FRET occurrence and distance relation. D represents donor fluorescent molecule, A represents acceptor fluorescent molecule, X and Y represent interacting molecules.

Green fluorescent Protein (GFP) was first discovered in the organism *Victoria aequorea* which is jelly fish (Shimomura, Johnson, & Saiga, 1962). Following this discovery, it is widely used in several research areas including cell biology research by protein tagging with gene fusions, which provides tracking of the protein and also this method adapted to FRET studies (Tsien, 1998).

Initially, during FRET studies, the pair of CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) was used especially in oligomerization studies of GPCRs (Milligan & Bouvier, 2005). Then, red and green pairs are selected for FRET studies, one of the examples of this is the dimerization research of GnRH receptors (Cornea, *et al.*, 2001). A red colored fluorescent protein mCherry and a green colored fluorescent protein EGFP (enhanced green fluorescent protein) which is derived from GFP, is thought to be a good FRET pair. The reason why this pair is a good FRET pair is: their

spectral properties and their overlapping regions are appropriate for the FRET. This means emission spectrum of EGFP overlaps with the excitation spectrum of mCherry, but also they have little crosstalk, thus reducing the false positives (Figure 1.6) (Albertazzi, Arosio, Marchetti, Ricci, & Beltram, 2009).

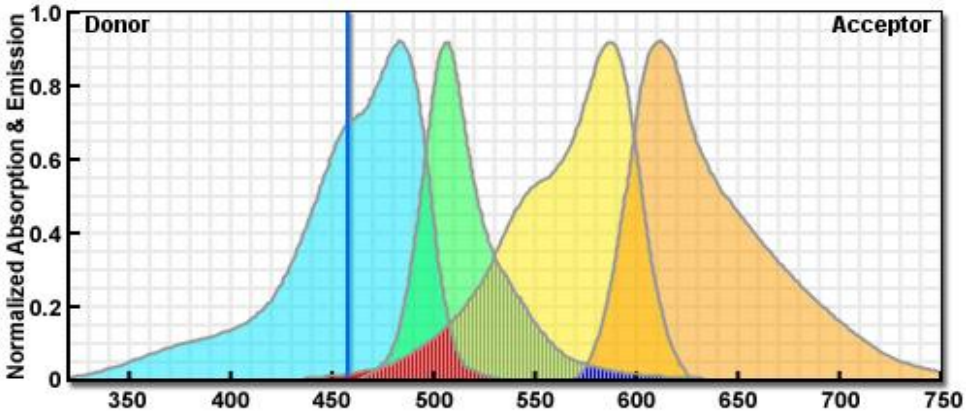

Figure 1.6. Spectral properties of EGFP and mCherry. Cyan and green colors represent EGFP spectra, yellow and orange colors represent mCherry spectra. Blue line represents the excitation wave length for FRET. Fluorescent Proteins JAVA Tutorial from MicroscopyU of Nikon® is used for drawing
(http://www.microscopyu.com/tutorials/java/fluorescence/fpfilterfinder/).

As mentioned in the previous sections a better FRET pair needs narrow overlapping region of emission. Figure 1.6 shows that EGFP and mCherry couple is very good considering this property because blue shaded area is emission overlap regions of couple and it is narrow. Moreover excitation spectra of acceptor and donor should not overlap much for preventing the excitation of acceptor while exciting the donor. Blue line in Figure 1.6 shows the excitation wavelength and at that wavelength mCherry is not

excited. One other point is donor emission spectrum should cover excitation spectrum of acceptor as much as it can which makes energy transfer easier, and the overlapping region between green area and yellow area in figure 1.8 represent this region which is enough for high FRET efficiency. By considering the information and the properties of EGFP and mCherry, they are a good FRET pair. FRET is more advantageous than the other methods in many aspects. One of the most important advantages is FRET allows detection of interaction in living cells. Also by FRET, localization studies, can be conducted which means we can detect the localization of interaction, in living cells (Herrick-Davis, Grinde, & Mazurkiewicz, 2004). Moreover, working with membrane proteins is difficult because they contain hydrophobic regions and hydrophilic regions together. Isolation of membrane proteins could cause conformational changes on them. However FRET is already applicable to membrane proteins without making any extra treatment.

1.6 Aim of the study

The aim of the present study is detecting Ste2p homodimerization in living cells with FRET method. Besides the main aim, dimerization location was investigated because in this study live cells were used without any fixation. We also tested the effect of environmental conditions such as presence or absence of alpha factor by using the constructs generated during this study.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Yeast strains

Yeast strain used in this experiment is DK102 which was kindly provided by Prof. Dr. Jeffrey M. Becker from University of Tennessee, Knoxville, USA.

DK102 cells are *MAT***a** type cells. They are also deficient in synthesizing uracil, tryptophan and they are BAR minus which means these cells do not have specific proteases for mating pheromone. All experiments including HALO assay and imaging are conducted using this strain.

Yeast extract-peptone-dextrose (YEPD) broth is the media that yeast cells were grown. Cells were grown at 30 °C and stored at 4 °C on agar plates for short period. Main stocks for long term storage were kept at -80 °C and they prepared by addition of 50 % glycerol as a cryoprotectant to a final glycerol concentration of 25 %. YEPD media was prepared by dissolving the components of media in dH_2O and sterilization was done by autoclave.

Selective media MLT (medium lacking Tryptophan), MLU (medium lacking Uracil) and MLTU (medium lacking tryptophan and Uracil) was used for selecting properly transformed yeast cells after transformation. Components of media are listed in Appendix A.

2.1.2 Bacterial strains

Competent *E.coli* strains, Top10 and DH5-α were used during this study. Luria Bertani (LB) broth and agar plates were used for growing bacterial strains. Components of media are listed in Appendix B. These components were dissolved in dH_2O, pH is adjusted to 7.4 by addition of drop wise 1N NaOH when required and autoclaved at 121 °C, for 20 minutes. After sterilization, the media were cooled for a while and then antibiotics were added to media for proper selection according to the following concentrations; 100 mg/mL of Ampicillin or 50mg/mL Kanamycin.

Cultures of both strains were incubated for growth at 37 °C incubators. Solid media were incubated in Nüve® branded incubators and liquid media need shaking so they were incubated in Zheiheng branded shaker incubator.

2.1.3 Plasmids

EGFP containing plasmid pEGFP-N2 was supplied by Dr. Henry Lester, California Institute of Technology, USA as generous gift. pBEC and pCL01 vectors were used as parental plasmids and contained the genes of Ste2p, donated by Prof. Dr. Jeffrey M. Becker, University of Tennessee Knoxville, USA.

2.1.4 Chemicals and materials

LA Taq polymerase was obtained from Takara Bio Inc. (Japan), Pfu ultra high fidelity polymerase was supplied from Stratagene (CA, USA), and Phire® Hot Start II DNA Polymerase was obtained from Thermo Scientific.

Restriction enzymes DpnI, BamHI, EcoRI and KpnI were obtained from NEB (New England Biolabs, Hertfordshire, UK). Alpha factor used in functional assays and imaging was supplied by Prof. Dr. Jeffrey M. Becker, University of Tennessee, Knoxville. Other chemicals that were used in media, buffer preparation and assays provided from Sigma-Aldrich Inc. (New York, USA) and Applichem (Darmstadt, Germany). *In vivo* cell imaging is conducted with LSM 510 (laser scanning microscope) at Bilkent University, UNAM.

2.2 Methods

2.2.1 $CaCl_2$ method for competent *E. coli* preparation

In first step Top10 *E.coli* cells were inoculated overnight in 50 mL LB media with 200 rpm and at 37 °C. Then 4 mL of it was taken into fresh 50 mL LB and incubated with same conditions for 3 hours. After that culture was incubated with ice for 15 minutes and centrifuged for 10 minutes at 4000 rpm. Supernatant was discarded and pellet was resuspended in 4 mL of 0.1 M $CaCl_2$. Next centrifugation process was repeated and pellet was resuspended in 0.1 M CaCl2/15 % glycerol solution. Final step was aliquot preparation and storing the aliquots at -80 °C. All equipment used in experiment was sterile and sterilization of autoclavable equipment was done by autoclave at 121°C for 20 minutes before beginning to the experiment. All experiment conducted with aseptic techniques.

2.2.2 Transformation of competent *E. coli*

Aliquots of *E.coli* cells prepared according to previous protocol were chilled on ice for 5 minutes for getting rid of ice crystals in tube. 1 µL (volume of

added DNA depends on DNA concentration) plasmid DNA was added on tube and it was gently mixed. Then cells incubated on ice for 30 minutes, after that heat shock at 42 °C was applied for 30-60 seconds to cells. Then cells placed on ice for 5 minutes and pre-heated 900 µl LB at 37 °C added on cells. It is followed by incubation of cells at 37 °C for 1 hour. Finally cells were centrifuged at 13000 rpm for 1 minute, 800 µL of supernatant discarded, remaining used for resuspension of cells and resuspended cells were inoculated on antibiotic selective (ampicillin or kanamycin) LB agar plates and incubated at 37 °C for 16 hours.

2.2.3 Ste2p fusion with fluorescent proteins and cloning to yeast

EGFP and mCherry cDNAs were amplified by PCR with primers that have extra DNA fragments at beginnings and ends of them. These overhanging regions are complementary to the STE2 at desired positions (304^{th} and 441^{st}). This amplification PCR is called as first PCR and a second PCR is done after first PCR with using first PCR products as primers and amplify whole plasmid containing STE2. Result of that second PCR, fusion of two genes occurs. Sequences of products are controlled with sequencing ones with correct sequence were transformed to DK102 cells of yeast to controlling receptors biological activity and also imaging (Figure 2.1).

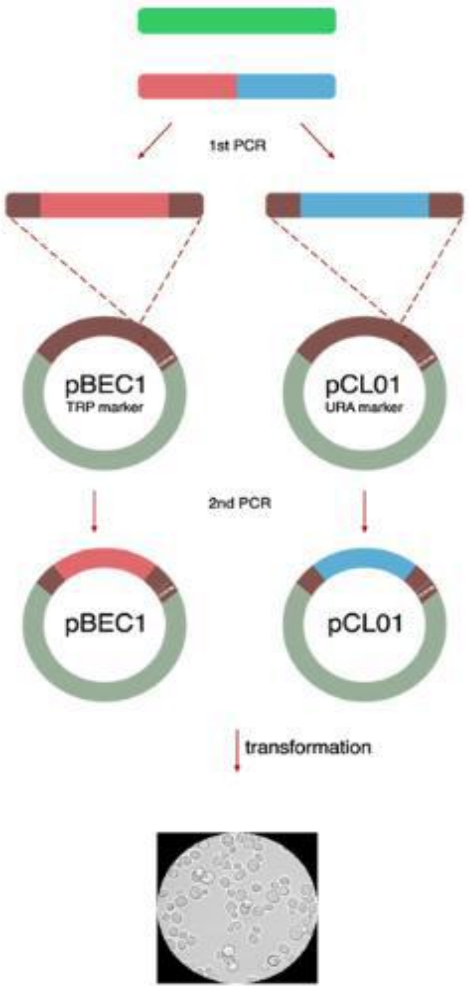

Figure 2.1. Fusion of fluorescent protein genes to STE2. Red color represent for mCherry, blue color for EGFP, brown for Ste2. 1st PCR arrow represents the amplification of fluorescent proteins with extra DNA fragments. 2nd PCR arrow represents the insertion of fluorescent proteins in the receptor in plasmids.

Two different positions of STE2 were fused with fluorescent protein gene sequences by using this method; these positions are 304th and 441st amino acids of the receptor all products are full length receptors.

2.2.4 Primers

Primers composed of two parts, one of them is located to 3' end of the primer and it is complementary to EGFP found in the plasmid pEGFP-N2 the remaining part is placed on the 5'end of the primer and it is complementary to desired insertion point sequence of the STE2, by that sequence, annealing of product of first PCR to Ste2p and amplification of it in second PCR occurs.

Lengths of the primers were changed between 40 to 50 bases. Tm (melting temperature) values of that primers show differences because Tm values dependent to GC content of the sequence and these primers had different GC contents. Primers are taken from Invitrogen (USA) and Alpha DNA (Montreal, Quebec). In table 2.1 lists of the primers are given.

In table 2.1 there are two sets of primers. They are classified according to their sequence similarity with the insertion points. Position 304 is named as that because amplified fluorescent protein had overhanging sequences complementary to insertion point between 304^{th} and 305^{th} amino acids of the Ste2p. And position 441 is named as that because amplified fluorescent protein had overhanging sequences complementary to insertion point between 441^{st} and 442^{nd} amino acids of the Ste2p. Same primers are used for both EGFP and mCherry amplification because design of the primers based on sequence similarities of that two fluorescent proteins.

Table 2.1. List of primers used in the amplification of fluorescent proteins and insertion to receptor. Primers were used for amplifying both mCherry and EGFP sequences with specific overhanging regions to defined positions of Ste2p.

Position	Primer	Sequence
304	Forward	CACGGCTGCTAATAATGCATCCAAAATG GTGAGCAAGGGCGAGGAG
	Reverse	GTAAAGTCTGAAGTAATTGTGTTTGTCTT GTACAGCTCGTCCATGCC
441	Forward	CAAGGACGACGATGACAAGACCGGTATG GTGAGCAAGGGCGAGGAG
	Reverse	GGCTGCTGCCGCTGCCGCGCGGCACCTT GTACAGCTCGTCCATGCC

2.2.5 PCR Reaction for Fluorescent Protein Amplification

Fluorescent proteins EGFP and mCherry are amplified with 3 different polymerases and designed primers with having overhang regions complementary to Ste2p sequence and optimized PCR conditions are described in table 2.2, 2.3, 2.4.

Table 2. 2. Pfu ultra PCR conditions.

Content	Volumes per reaction
dH_2O	36 µL
Pfu Buffer (10X)	10 µL
dNTP (25 nM)	0,5 µL
Forward Primer (10ng/ µL)	1 µL
Reverse Primer (10ng/ µL)	1 µL
Template (100ng/ µL)	1 µL
Pfu ultra polymerase	0,5 µL
Total	50 µL

Cycle Parameters			
Pre-denaturation	94 °C	5 min.	
Denaturation	94 °C	30 sec.	35 cycles
Annealing	53 °C	45 sec.	35 cycles
Extension	72 °C	1min	35 cycles
Final extension	72 °C	5 min.	

Table 2. 3. *Taq* DNA polymerase PCR conditions.

Content	Volumes per reaction
dH_2O	36 µL
Pfu Buffer 10X	10 µL
dNTP (25 nM)	0,5 µL
Forward Primer (10ng/ µL)	1 µL
Reverse Primer (10ng/ µL)	1 µL
Template (100ng/ µL)	1 µL
Pfu ultra polymerase	0,5 µL
Total	50 µL

Table 2. 4 cont'd. *Taq* DNA polymerase PCR conditions.

Cycle Parameters			
Pre-denaturation	94 °C	5 min.	
Denaturation	94 °C	30 sec.	35 cycles
Annealing	53 °C	45 sec.	35 cycles
Extension	72 °C	1min	35 cycles
Final extension	72 °C	5 min.	

Table 2. 5 Phire® Hot Start II DNA Polymerase PCR conditions

Content	Volumes per reaction
dH_2O	36 µL
Pfu Buffer 10X	10 µL
dNTP (25 nM)	0,5 µL
Forward Primer (10ng/ µL)	1 µL
Reverse Primer (10ng/ µL)	1 µL
Template (100ng/ µL)	1 µL
Phire® Hot Start II DNA Polymerase	0,5 µL
Total	50 µL

Cycle Parameters			
Pre-denaturation	98 °C	5 min.	
Denaturation	94 °C	30 sec.	35 cycles
Annealing	53 °C	45 sec.	35 cycles
Extension	72 °C	1min	35 cycles
Final extension	72 °C	5 min.	

Controlling the sizes of PCR results were done by agarose gel electrophoresis. 1 % agarose gel was prepared for this purpose by dissolving proper amount of agarose in 1X TBE and heating it in microwave. When it was cooled around 55 °C EtBr added to solution. This solution was poured to tray and cooled until it was completely solidified. Bubbles should be avoided while pouring the solution to tray. Solidified gel was placed into the electrophoresis chamber and TBE buffer was added to chamber until it covers the gel completely. 6X loading buffer mixed to samples with the ratio of 1/5 and final concentration of buffer become 1X. Then samples were loaded to wells. First well was separated for DNA ladder as standard. Final step was running the gel at 100V for 30 minutes. The results were pictured under UV light. Proper samples were selected and cut from the gel for gel isolation.

2.2.6 Gel isolation

Fermentas GeneJET$_{TM}$ Gel isolation Kit and protocol used during this study for gel extraction.
Concentrations of isolated inserts were measured with Nanodrop 2000.

2.2.7 Second (insertion) PCR

Fluorescent proteins placed into the proper position of Ste2 by second PCR. First PCR products, which are fluorescent proteins with flanking sequences at both ends, are used as primer for that PCR. pBEC and pCL01 plasmids containing Ste2p are used as template for that reaction. Ratio of plasmid to template is 5:1 and also high fidelity DNA polymerases (phire, phusion or pfu turbo) are used to prevent mutations. Final products of PCR are plasmids carrying Ste2p with fluorescent labeling at proper position.

2.2.8 DpnI treatment

In *E. coli* like most of other bacteria CH_3 groups are added to DNA at specific sequences to make discrimination between native and foreign DNA by methylase. DpnI recognize the sequence GATC at which adenine is methylated and digest the DNA between adenine and thymine. By that reaction the templates are used in PCR digested.

DpnI treatment to second PCR products are conducted according to using defined mixture composition in table 2.5, and incubated at 37 °C overnight. After incubation 2-5 μL of digested product amplified by *E.coli* transformation which is defined earlier in 2.2.2 and selection is done on ampicillin plates. 5-10 colonies selected for screening. For that purpose colonies are grown for miniprep isolation.

Table 2. 6. DpnI mixture for removing the bacterial originated DNA from the PCR product.

Content	Volumes per reaction
Second PCR Product	17 μL
Dpn I Enzyme	1 μL
Buffer-4 (Appendix C)	2μL

2.2.9 Plasmid isolation

Fermentas GeneJET$_{TM}$ Plasmid Miniprep Kit and protocol used during this study for plasmid isolation.
Concentrations of isolated plasmids were measured with Nanodrop 2000.

2.2.10 Size control with restriction enzymes

BamHI and EcoRI restriction enzymes were used in restriction control part of that study which was purchased from New England Biolabs Inc. Double digestion is applied to plasmids. For that 1 μg of DNA samples were incubated with 1 μL (1 unit) of restriction enzyme and 1X BSA for 2 hours at 37 °C. After restriction digestion size of the products were controlled with agarose gel electrophoresis which is described before.

2.2.11 Sequence control with sequencing

Sequences of all constructs were confirmed by DNA sequencing which was carried out in Refgen (Ankara, Turkey) and MCLAB (California, USA)

2.2.12 Yeast transformation with modified LiAc method

LiAc/ss-DNA/PEG transformation method was used for high efficiency transformation (Gietz & Schiestl, 1995). Solutions which are used in that method prepared under aseptic conditions for that sterilization is applied either by filter or autoclave. List of the solutions' recipes was given in the Appendix C. Before starting transformation protocol, DK102 cells were grown overnight in 5 ml of proper medium at 30 °C with shaking. After that cells were counted and inoculated in 50 ml medium to arrange cell density to $5x10^6$ cells/ml. These cultures were incubated again until density of the cells was reached to $2x10^7$ cells/ml. Culture was collected by centrifugation at 4000 rpm for 5 minutes. After supernatant was discarded, cells were resuspended in 25 ml sterile dH_2O then centrifuged again for washing. Water was discarded after centrifugation and cells were resuspended in 1 ml. Next transferred to 1.5 ml eppendorf tube and centrifuged at top speed

for 15 seconds. Centrifugation was followed by removal of LiAc with micropipette. Cells were resuspended in 400 µl 100 mM LiAc solution and 50 µl of aliquots were taken. Cells were centrifuged again and LiAc solution removed then; 240 µl PEG (50 % w/v), 36 µl 1.0 M LiAc, 25 µl salmon sperm DNA (boiled for 5 minutes before being used), 50 µl water and plasmid DNA (0.1 - 10µg) were added with that order. Tube was vortexed until pellet completely resuspend in the mixture and incubated for 30 minutes at 30 °C. After incubation heat shock at 42 °C for 20-25 minutes was applied next tubes were centrifuged at 8000 (or max) rpm for 30 seconds. Supernatant was removed and cells were dissolved in 1 ml dH_2O. After that 200 µl of solution taken and placed to selective media agar plates. Plates were incubated at 30 °C for 2 days. Single colonies were selected and grown for further studies.

2.2.13 Functional assay

DK102 cells expressing the tagged (EGFP, mCherry and both) Ste2p were grown at 30 °C overnight. After that cells were harvested and washed for 3 times with sterile water and resuspended at a final concentration of $5x10^6$ cells/ml. Next cells were mixed with 4 ml 1.1% noble agar and poured as a top agar lawn onto MLT/MLU medium agar plates. Filter disks (BD, Franklin Lakes, NJ) were placed on the top agar and impregnated with 10 µl of alpha-factor pheromone at various concentrations. Plates were incubated at 30 °C for 20-30 hours and clear zones (halos) were observed around the disks.

2.2.14 Imaging and image analysis

For image acquisition, yeast cells were grown at 30 °C overnight. After that subculture in 5 ml fresh media and incubated for 3 hours. The cells were

diluted for imaging. Sterile and fresh culture media was used for dilutions. For detection of FRET in live cells, observations are done with Zeiss LSM510 confocal microscope. All images have been taken by using same settings. Only changes were done to images about the image size, brightness and contrast. Images were analyzed by using software imageJ v1.46 and its plug-in PixFRET.

2.2.15 Statistical analysis

Statistical analyzes were done with using software Graphpad Prism5. t-test derivative Mann-Whitney test was applied to compare different conditions. Mann-Whitney test was selected as the efficiency of this test is better than student t-test for non-parametric samples that are not following a normal distribution.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Construction of Ste2p Fusion Proteins with Fluorescent Tags

EGFP and mCherry proteins were integrated to our receptor by two tandem PCRs which was shown in Figure 2.1 in chapter 2. First PCR was conducted for amplifying EGFP and mCherry sequences with 25-26 bp extra DNA fragments. Those DNA fragments were added to fluorescent proteins using the primers containing the sequences that given in Table 2.1. DNA fragments of the first PCR products are homologous to Ste2p from the desired regions (304 and 441) that are shown with blue arrows in figure 3.1. pEGFP-N2 was used as source for EGFP gene and mCherry gene was taken from the constructs (A2AR) of Gökhan ÜNLÜ's master thesis studies.

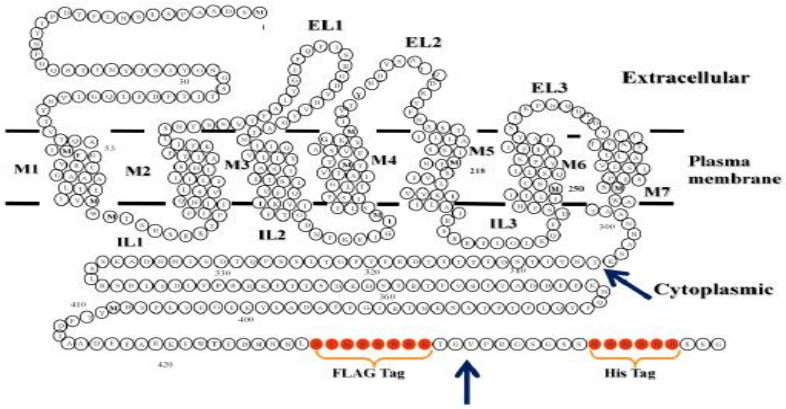

Figure 3.1. Diagram of Ste2p (yeast α-factor receptor). Blue arrows represent insertion positions of fluorescent proteins. First arrow is 304th position and the second (between FLAG tag and His tag) is 441st positions of the receptor.

First PCR products run on agarose gel which shown in figure 3.2. First lane was loaded with 1 kb plus DNA ladder. Second lane for EGFP 304, third lanes was loaded with EGFP 441. Last two lanes were containing PCR product of mCherry with same order of EGFP lanes. Product sizes of both fluorescent proteins were around 800 bp. Two lanes for one sample used because whole PCR product could be loaded at least two lanes and whole product was desired for gel isolation.

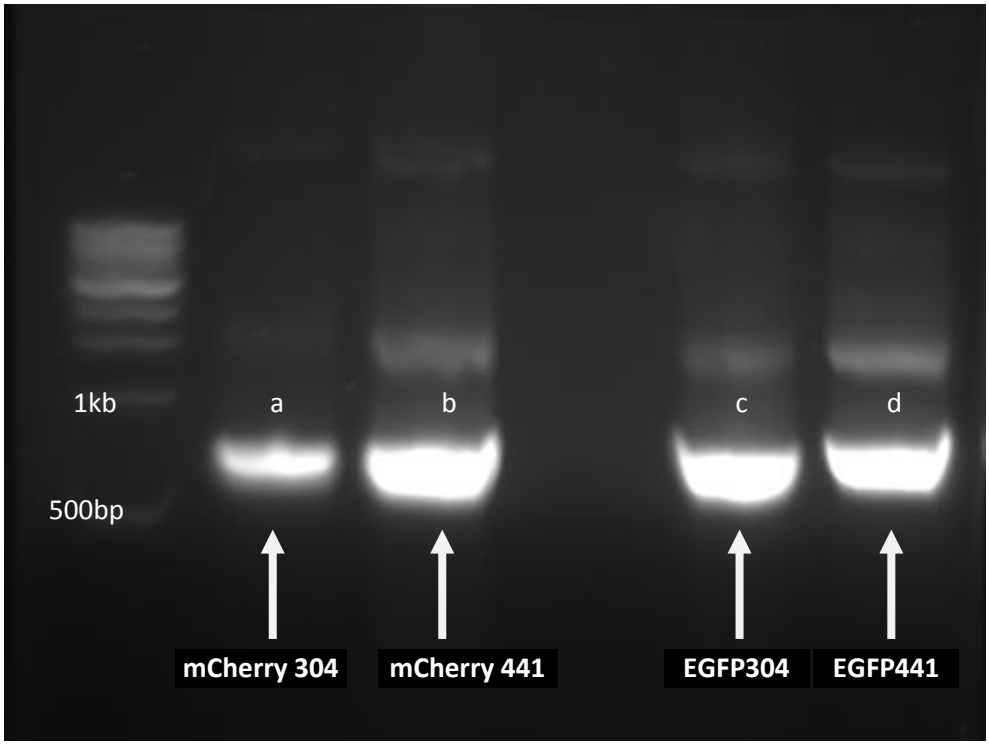

Figure 3. 2 Agarose gel image of PCR products. a) mCherry that has extra DNA part which are complementary to 304th aa position of the receptor. b) mCherry that has extra DNA part which are complementary to 441st aa position of the receptor. c) EGFP that has extra DNA part which are complementary to 304th aa position of the receptor. d) EGFP that has extra DNA part which are complementary to 441st aa position of the receptor.

Then the products were cut out from the gel for gel isolation and products were isolated with the Fermentas GeneJET$_{TM}$ Gel Isolation Kit. Next concentrations were measured with nanodrop. Concentrations are given in Table 3.1 below.

Table 3.1. Concentrations of isolated first PCR products from the gel.

Type	Concentration
EGFP304	194.8 ng/µl
EGFP441	101.9 ng/µl
mCherry304	70.3 ng/µl
mCherry441	68.6 ng/µl

According to those first PCR products' concentrations second PCR composition calculated. Ratio of first PCR product to template was 5:1. Then first PCR products were used as primer for the second PCR. Template of the second PCR was plasmids (pBEC and pCL01) that containing Ste2p gene with different selection properties. Final product of that two PCR was Ste2p gene with fused fluorescent tags at desired positions. To get rid of template plasmid products were treated with DpnI. Because DpnI cut methylated DNA and by that template was eliminated from the product. Results of that treatment, products contain only PCR products.

3.2 Amplification and selection of correct plasmids

PCR products transformed to competent *E.coli* cells. Both plasmids contain resistance genes for ampicillin so cells were grown on ampicillin containing agar plates. Resistance to ampicillin was first selection then colonies were screened with colony PCR. For that samples of all colonies were used as

template for PCR and first PCR primers used as primer. Result of colony PCR was shown in figure 3.3.

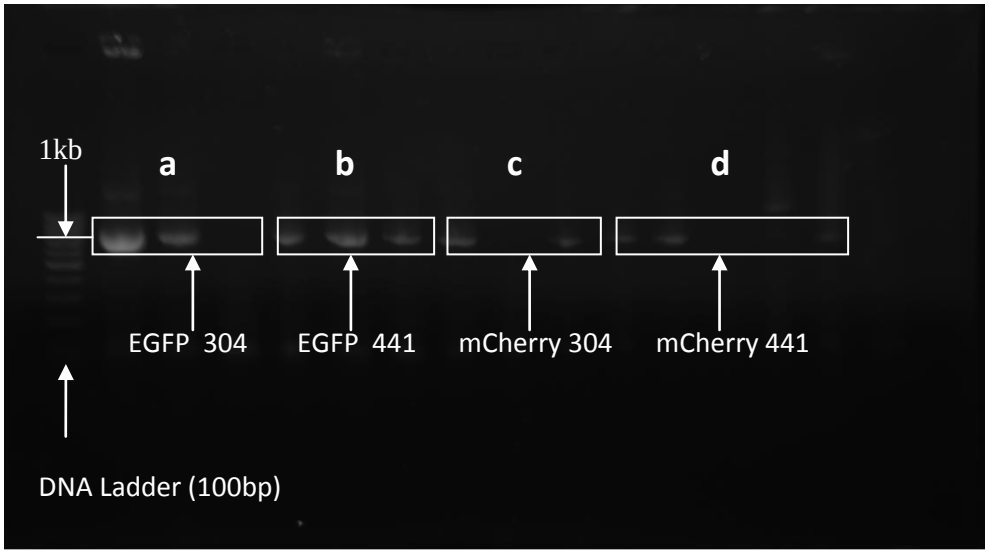

Figure 3.3. Colony PCR results first lane is the DNA ladder (100bp) a) Colonies selected for testing the existence of EGFP at 304th position after transformation. b) Colonies selected for testing the existence of EGFP at 441st position after transformation. c) Colonies selected for testing the existence of mCherry at 304th position after transformation. d) Colonies selected for testing the existence of mCherry at 441st position after transformation.

Colony PCR was a fast way of screening because it eliminates the time for cell growth. According to figure 3.3 positive colonies were selected and grown for plasmid isolation. Next day plasmids were isolated from the *E.coli* with Fermentas GeneJET$_{TM}$ Plasmid Miniprep Kit and concentrations of the plasmids were measured with nanodrop and given in table 3.2.

Table 3. 2 Concentrations of isolated plasmids.

Type of plasmid	Concentration
mCherry304 pNED	345.2 ng/µl
mCherry441 pNED	281.2 ng/µl
EGFP304 pBEC	341.3 ng/µl
EGFP441 pBEC	353.7 ng/µl
mCherry304 pBEC	280,5 ng/µl
mCherry441 pBEC	479.1 ng/µl
EGFP304 pNED	368.8 ng/µl
EGFP 441 pNED	207.6 ng/µl

After plasmid isolation one more control of the plasmids were done with digestion. EcoRI and BamHI enzymes were selected for that experiment. Because they cut the plasmid only at one site and by that cut Ste2p part of the plasmid separates from the plasmid. Gel image of that trial was given in figure 3.4.

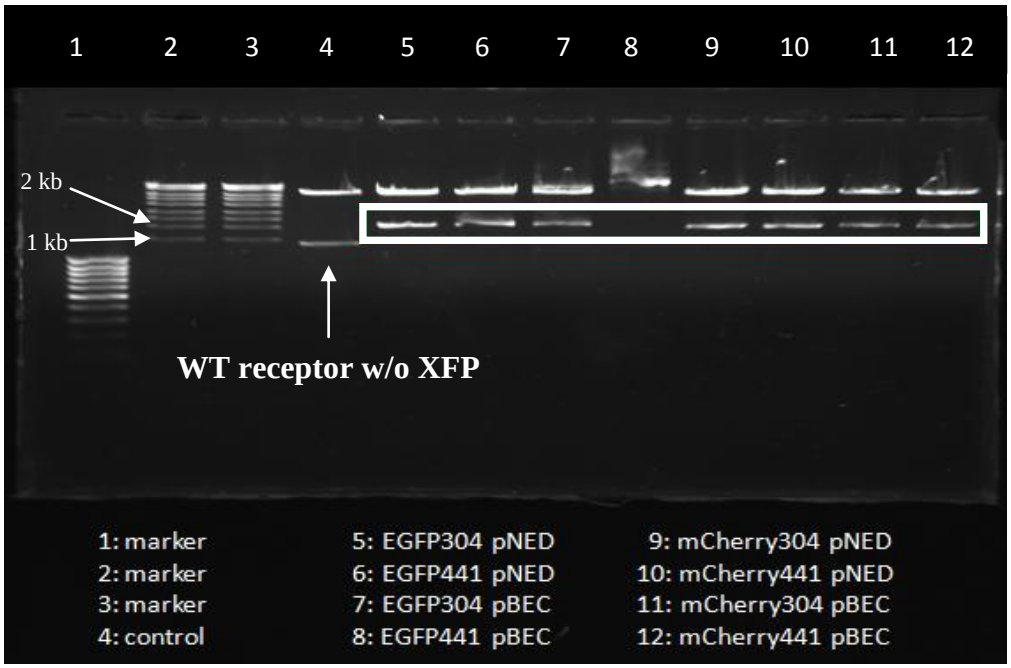

Figure 3. 4 Digestion results of the plasmids. 1st lane marker is low range 2nd and 3rd lane markers are high range at 4th lane wild type STE2 bearing plasmid loaded which does not contain sequences encode for fluorescent protein.

3.3 Transformation to yeast control of functionality of receptors

Selected plasmids were transfected to yeast (strain DK102) by a modified LiAc method. Selective media were used for selection of proper plasmid containing transformants. pCL01 plasmid transfected cells were grown on medium lacking uracil (MLU) and pBEC2 transfected cells were grown on medium lacking tryptophane (MLT). For FRET studies both plasmids were co-transfected to single cell at once or with repeating transfection which mean one transfection was followed by other. After transfection, cells were grown for two days. Then functionality of the receptor was tested using the HALO assay and clear zone diameters were recorded. That assay based on the cells response to pheromone, so pheromone loaded discs were placed on

the plate, in top agar and cell growth stops at G1 phase, as a result of that, clear zones around the discs were formed. This assay was performed to detect receptors will be functional after fused with fluorescent protein. And representative figure is presented below.

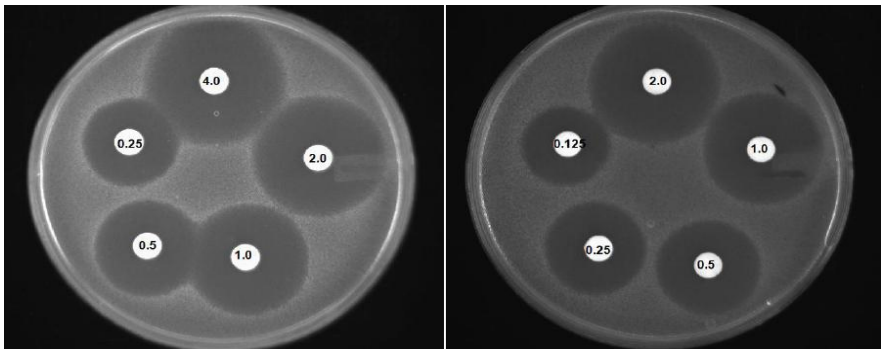

Figure 3.5. Representative image of Halo assay. Various amounts of pheromone (0.0125- 0.1 μg) loaded to filter discs.

Halo radius was measured after growth of cells and data are given in Table3.3. Graph of all types of transfection were drawn. After that their linear regression analysis was done with using Graphpad Prism 5 and according to that analysis slopes of all cell types are not significantly different from each other (Figure 3.6). Also another plate for negative control was tested and zones around the discs could not be observed. This mean all of the receptors are functional after being fused with fluorescent proteins.

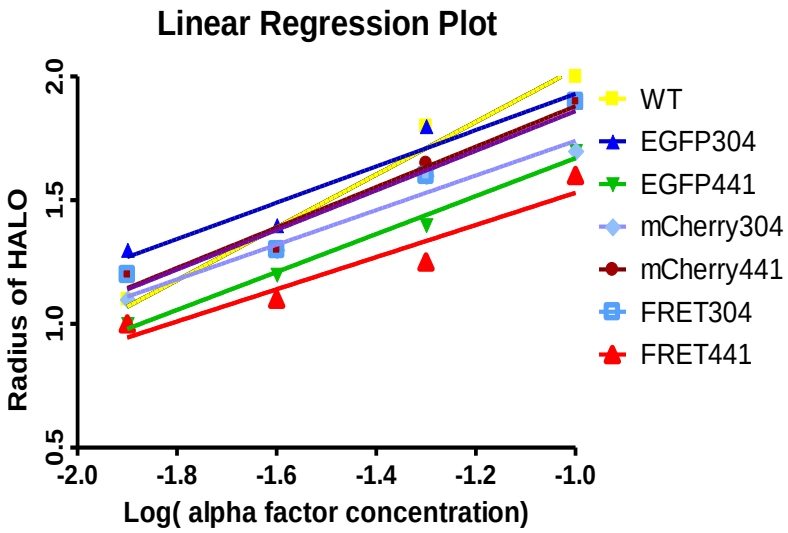

Figure 3. 6 Linear regression plot of the HALO diameters of each type of transfection. By that plot their slopes were tested whether they are similar or not for testing the effect of fluorescent protein integration on functionality.. According to analysis slopes were not significantly different from each other. x axis represents the logarithmic concentration of alpha factors loaded to discs, y axis represents the radius of HALO diameters.

Table 3. 3 Halo assay measurements left column represents the type of receptor first row represent the concentration of loaded alpha factor to discs. Measured lengths are the radius of zones around the discs.

Type /concentration of pheromone	**0.0125nM**	**0.025nM**	**0.05nM**	**0.1nM**
WT	1.1 cm	1.3 cm	1.8 cm	2 cm
EGFP304	1.3 cm	1.4 cm	1.8 cm	1.9 cm
EGFP 441	1 cm	1.2 cm	1.4 cm	1.7 cm
mCherry 304	1.1 cm	1.3 cm	1.6 cm	1.7 cm
mCherry441	1.2 cm	1.3 cm	1.65 cm	1.9 cm
FRET304	1.2 cm	1.3 cm	1.6 cm	1.9 cm
FRET 441	1 cm	1.1 cm	1.25 cm	1.6 cm

After that point whether cells were expressing fluorescent protein or not were tested with confocal microscope. Single transfected cell images were also required for FRET calculations. Representative images for single transfection to 304^{th} and 441^{st} positions with fluorescent protein sequence bearing plasmids are given in Figure 3.7.

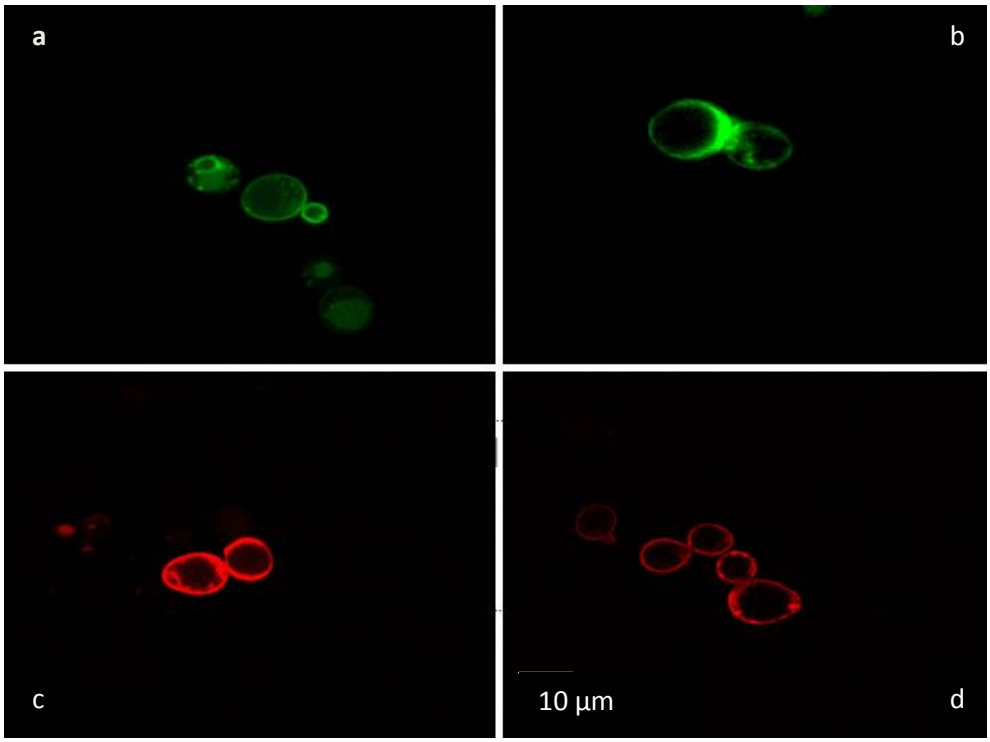

Figure 3.7. Single plasmid transfected yeast images a) Representative image for EGFP304 b) Representative image for EGFP441 c) Representative image for mCherry304 d) Representative image for mCherry 441. This images were artificially colored by using software imageJ and colors selected based on the fluorescent proteins' real colors. Brightness of the color indicates higher signal intensity. More images are presented in Appendix F.

3.4 FRET analysis

Transfected cells were investigated with LSM510 confocal microscope by using multi track settings as described in Figure 3.8.

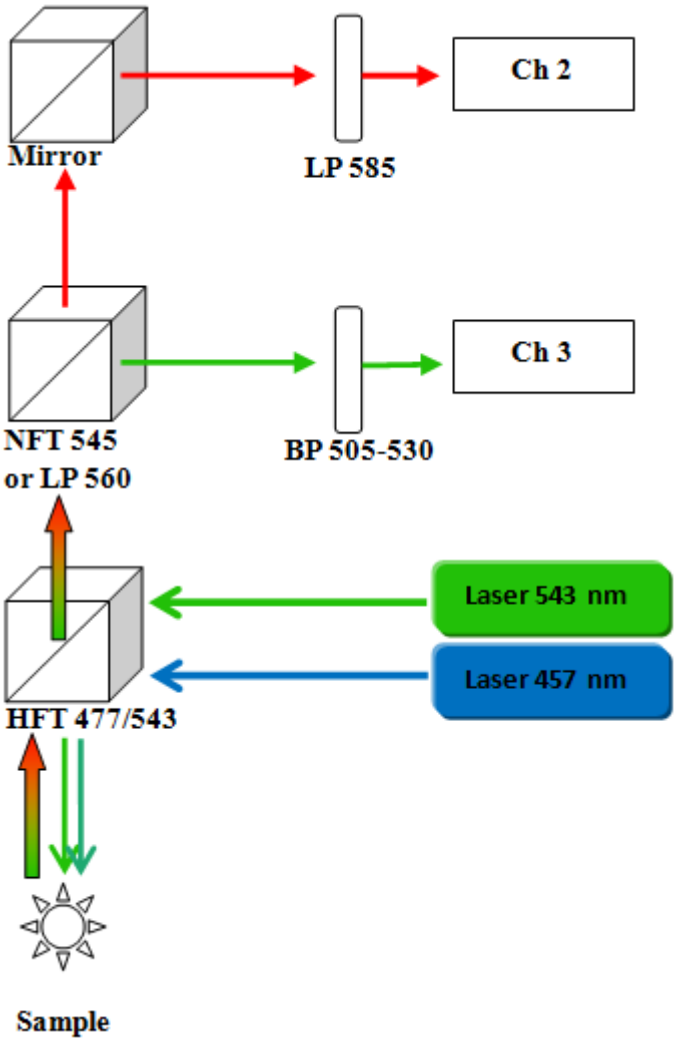

Figure 3.8. Multi track settings of microscope a) For green and FRET signals b) For red signals

With laser 457 EGFP containing samples could be excited. Detection of EGFP signal was done with channel 3 and channel 2 detects FRET signal.

With Laser 543 mCherry containing cells were excited according to that red signal could be collected from channel2 and channel 3 in that setting detects EGFP signal. In Figure 3.9 spectral properties of EGFP, mCherry and channel widths are given.

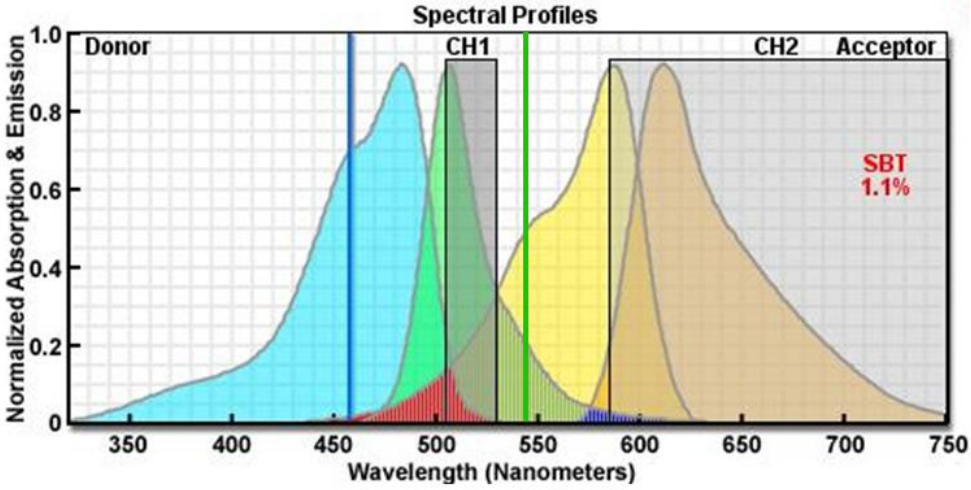

Figure 3. 9 Spectral properties of fluorophores and channel widths. CH1 is the green data collected zone CH2 is the red data collected zone.

Images were taken according to these settings and were analyzed using imageJ v1.46 and plug in PixFRET. For analysis image stacks were prepared. There are three types of stack; DB, AB and FRET. DB stack contains 2 images those are Ch3-T1 for EGFP signal and Ch2-T1 for FRET signal (Ch: channel, T1: track 1 which is first one in Figure 3.8), AB stack contains Ch3-T2 for mCherry signal and Ch3-T1 for FRET signal and FRET stack contains 3 images Ch3-T2 for mCherry only, Ch2-T1 for EGFP only and Ch3-T1 for FRET signal. After that by PixFRET acceptor and donor bleed through calculations done for FRET measurement. FRET calculations were done with FRET efficiency option in PixFRET. FRET efficiency directly related to distance between fluorophores and calculated

with equation: $E=1/1+(r/R_0)^6$. After that calculation FRET efficiency images were artificially colored with lookup table option 5-ramps and the representative image is shown in Figure 3.10.

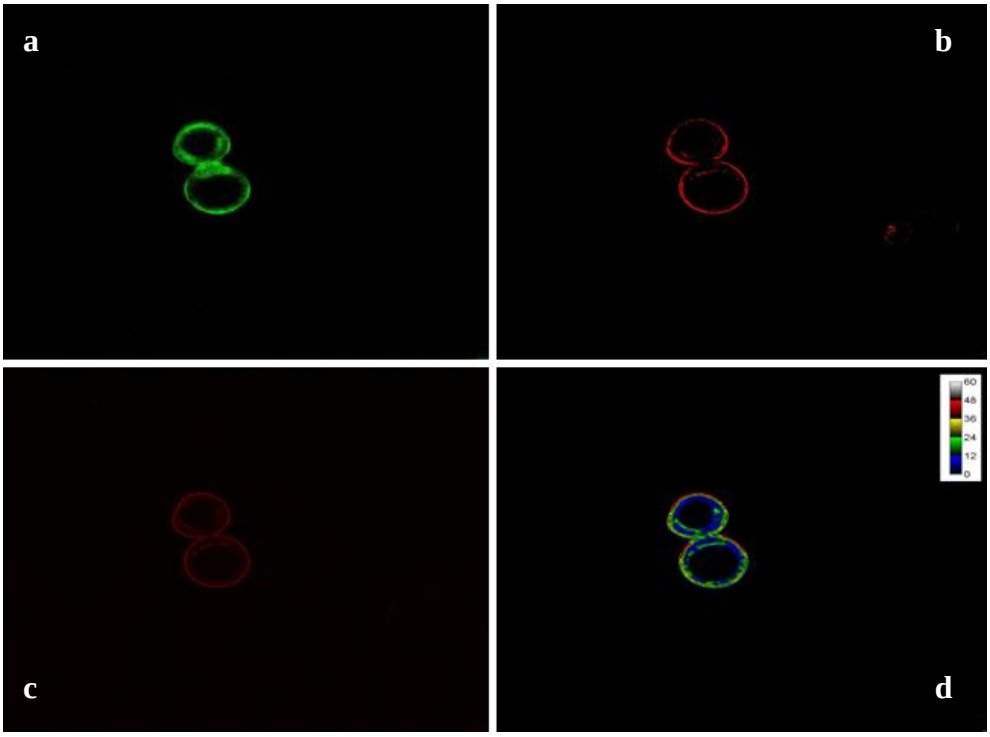

Figure 3. 10 Representative image of artificially colored FRET efficiency image. a) Ch1-T1 which collect only green signal, only green fluorescent protein was excited. b) Ch2-T2 which collect red signal, only red fluorescent protein was excited. c) Ch2-T1 collect FRET signal, green fluorophores were excited and red signal was collected. d) FRET efficiency image, each color in that artificial coloring represents a different FRET efficiency range defined at bar. FRET efficiency increased by following the order, blue (0-12), green (13-24), yellow (25-36), red (37-48) and grey (49-60). Images in a, b, and c were used for preparing the stack images that was needed for FRET efficiency calculations. Increased FRET efficiency pixels mainly observed at membrane.

After coloring the image, FRET regions were selected and histogram of that image was obtained from the imageJ and representative histogram data were given in Figure 3.11.

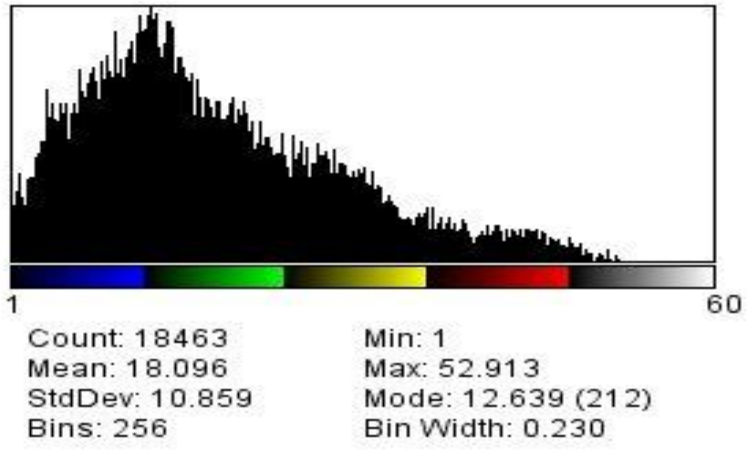

Figure 3. 11 Representative histogram image blue color zone is in the range between 1-12 green color zone is in the range between 12-24 yellow color zone is in the range between 24-36 red color zone is in the range between 36-48 grey color zone is the range 48-60

Means of all images were recorded for statistical analysis. For FRET 304, FRET304+alpha factor and FRET441 20 cells were recorded. For FRET441+alpha factor 16 cells were recorded. Data were given in Appendix D.

After this point by using software c statistical analysis were done. Data sets were compared with each other two by two. Results of those tests were given below.

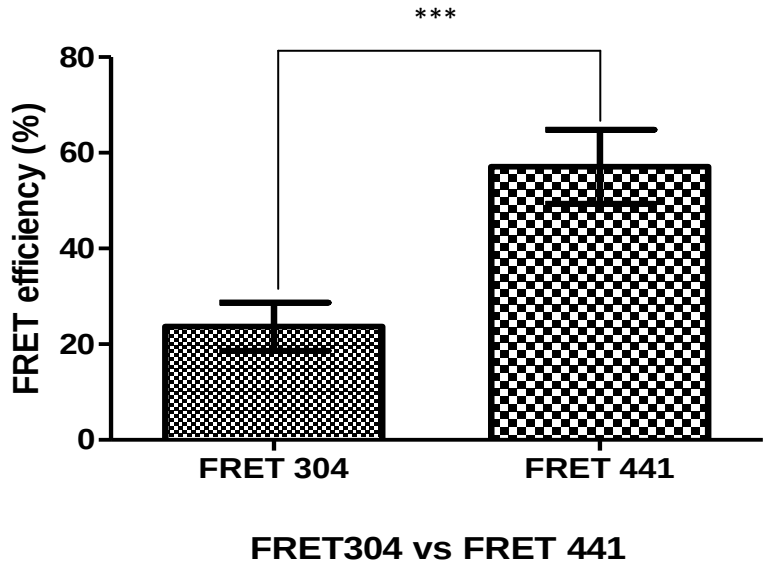

Figure 3.12. Graph of two different labeling positions of Ste2 FRET efficiency means. Mean of 304 is 23.67 and mean of 441 is 57.12 Their Mann-Whitney test results were significantly different from each other with p value <0.0001

This result indicates that 441st position fret efficiency was increased according to 304th position. This mean fluorophores at 441st position were closer to each other than 304th position. It could be caused by tip of tails approach to each other at that position.

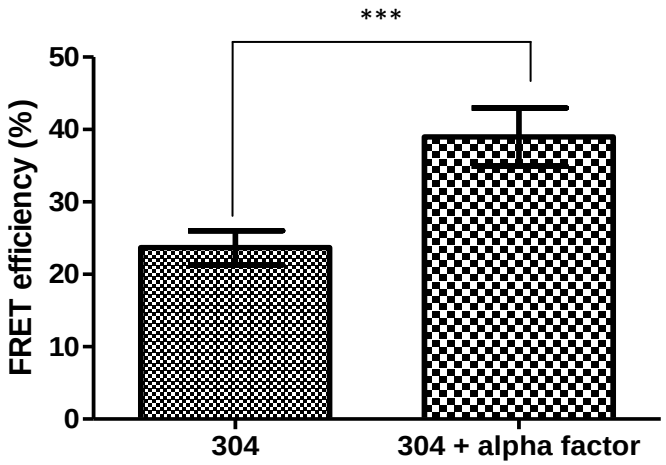

Figure 3.13. Graph of effect on alpha factor on means of Ste2 FRET efficiency at 304th position. Mean of 304 is 23.67 and mean of 304+alpha factor is 38.96 Their Mann-Whitney test results were significantly different from each other with p value < 0.0001

In Raicu's 2005 study effect of ligand was tested at 304th position with similar ways and conclusion of that study was ligand has no effect on the dimerization but results of this study for 304th position indicates an increase when cells were incubated with pheromone (Raicu V., 2005). Main reason of that could be caused by the type of receptor that they used. They used truncated forms of the receptor but in this study full length Ste2p was used.

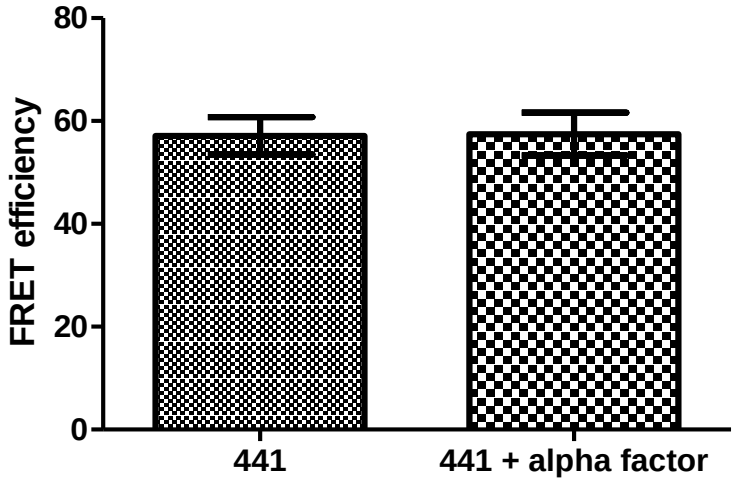

Figure 3. 14 Graph of effect on alpha factor on means of Ste2 FRET efficiency at 441th position. Mean of 441 is 57.12 and mean of 441+alpha factor is 57.44. Their Mann-Whitney test results were not different from each other with p value = 0.9873

According to that result alpha factor incubation has no effect on FRET efficiency. This might be caused by several reasons. One of them efficiency was already high and it could not cause a detectible difference. Other reason could be conformational changes after ligand binding could not lead any change in distance of fluorophores.

CHAPTER 4

4. CONCLUSION

In conclusion this study was designed for detecting homodimerization of the receptor Ste2p *in vivo.* Ste2p is a model GPCR which represents a large family of receptors important for signal transduction mechanisms and sensing environment.

By this study a model for *in vivo* investigation of GPCRs was optimized. Previous studies prove their dimerization after isolation of the receptor Ste2p (Hebert *et al*., 1996). Isolation of this kind of integral membrane proteins could be problematic due to their composition, as they contain hydrophilic and hydrophobic parts together. But results of this study are supportive to previous studies.

In this study, two different positions were labeled those are 304^{th} position and 441^{st} position. In 441^{st} position FRET efficiency was higher compared to 304^{th} position. This means in 441^{st} position, fluorescent proteins were closer to each other than 304^{th} position in the resting state.

And all conditions were tested during this study indicated higher FRET at membrane and lower FRET inside the cells. This result minimizes the probability of signal that is collected from dimerization of fluorescent proteins themselves. This result also implies tails of two receptors are getting closer to each other at their end.

Effect of pheromone on dimerization was also tested in this study for both positions. In 304th position pheromone incubated cells have higher FRET efficiency which means fluorophores getting closer due to pheromone response of receptor which implies a conformational change of receptor. In 441st position no significant change was observed after pheromone addition indicating the tails were relatively more stable compared to TM7 upon interaction with the pheromone.

According to these results dimerization of Ste2 receptor indicated *in vivo* but this results need to be confirmed with several experiments. Mutating interaction points of the receptor and investigation of change on FRET efficiencies according to mutations are one of the possible future applications where this optimized system can be used.

Using the methods optimized in this study, higher states of oligomerization structures of Ste2p receptor could be studied. Localization of dimerization could be detected by using several organelle markers. Moreover live imaging could be done and effects of various external effectors such as drugs on receptor dimerization can be tested by using live cells.

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APPENDICES

APPENDIX A

Yeast media components

Table A. 1 Composition of dropout mix (stock) for Media Lack of Tryptophane, Uracil,Tryptophane and Uracil (MLT, MLU and MLTU)

Component	**Final concentration (g/L)**
Adenine Sulfate	0.058
Arginine HCl	0.026
Asparagine	0.058
Aspartic Acid	0.14
Glutamic Acid	0.14
Histidine HCl	0.028
Isoleucine	0.028
Leucine	0.083
Lysine	0.042
Methonine	0.028
Phenylalanine	0.69
Serine	0.52
Threonine	0.28
Tyrosine	0.042
Tryptophane*	0.03
Valine	0.21
Uracil*	0.028

***** These aminoacids were cancelled from the formulation of dropout mix one by one or together due to the intended selectivity.

Medium composition:

20 g/L Glucose
10 g/L Casamino Acids
6.7 g/L YNB without Aminoacids
1.8 g/L Dropout mix

15 g/L Agar is added to the media after autoclave sterilization for solid agar plate preparation.

YEPD Media Composition:

10 g/L Yeast extract
20 g/L Peptone
20g/L Glucose

For YEPD agar 15g/L agar is added to the mixture and sterilized by autoclaving.

APPENDIX B

Bacterial media components

Luria-Bertani (LB) Medium:

10 g/L Tryptone
5 g/L Yeast extract
5 g/L NaCl
15 g/L Agar is added to the media after autoclave sterilization for solid agar plate preparation.

Super Optimum Broth with Catabolite repression (SOC):

20 g/L Tryptone
5 g/L Yeast Extract
0.5 g/L NaCL
0.186 g/L KCl
0.952 g/L MgCl2
2.408 g/L MgSO4
3.603 g/L Glucose

pH adjusted to 7.0 and medium was sterilized by autoclaving at 121 °C for 20 minutes.

APPENDIX C

Solutions and buffers

6X DNA Loading Dye Ingredients

10 mM Tris-HCl (pH7.6)
0.03 % Bromophenol Blue
0.03 % Xylene Cyanol FF
60 % Glycerol
60 mM EDTA

10X Tris-Borate-EDTA (TBE) Buffer

108 g/L Tris Base (890 mM)
55 g/L Boric Acid (890 mM)
40 ml/L EDTA (20 mM)
All ingredients was added to 800 ml of dH2O and volume was adjusted to 1L. For gel electrophoresis, the solution was diluted 1:10.

1X NEB Buffer 4

20 mM Tris-acetate
50 mM Potassium acetate
10 mM Magnesium acetate
1 mM Dithiothreitol
pH 7.9 at 25 °C

Single-stranded Carrier DNA (2 mg/ml):

200 mg of salmon sperm DNA (DNA Sodium Salt from salmon testes, Sigma D1626) was dissolved in 100 ml of TE buffer (Sigma, #93283) on a magnetic stirrer for 3-5 hours. 500 µl aliquots of the solution were prepared and stored in -20 °C.

1.0M Lithium Acetate Solution:

Prepared as stock solution in distilled deionized water (ddH2O). The solution was filter sterilized and final pH was adjusted to 8.4 – 8.9. Required dilution was made to obtain 100 mM Lithium acetate solution.

Polyethylene glycol (PEG 50%):

50 mg of the polyethylene glycol (PEG) (Sigma, #P3640) was mixed with 35 ml of ddH2O on a magnetic stirrer until it solved completely. Volume was adjusted to 100 ml and the solution sterilized by autoclave. For long term storage the cap of the bottle sealed with parafilm to prevent evaporation of water and increase in PEG concentration.

APPENDIX D

FRET efficiency data

Table A.2. FRET data

FRET 304				**FRET 441**		
	Count	**Mean**			**Count**	**Mean**
cell 1	7420	18,123		cell1	15704	50,272
cell 2	5395	22,489		cell2	7306	55,107
cell 3	1791	25,684		cell3	4359	45,268
cell 4	4403	26,143		cell4	3027	40,887
cell 5	2877	30,285		cell5	1200	68,765
cell 6	3171	29,959		cell6	1467	56,812
cell 7	5644	33,604		cell7	5042	56,214
cell 8	1652	18,721		cell8	572	69,527
cell 9	862	18,072		cell9	510	63,584
cell 10	2726	15,955		cell10	4190	49,912
cell 11	1137	22.617		cell11	2838	57,383
cell 12	1729	13,608		cell12	1980	59,106
cell 13	2118	22,032		cell13	464	64,817
cell 14	836	27.572		cell14	934	70,715
cell 15	1198	23,265		cell15	1829	56,276
cell 16	1594	23,757		cell16	2361	57,218
cell 17	849	26,867		cell17	1737	59,858
cell 18	1287	23,215		cell18	4224	55,307
cell 19	1097	27,503		cell19	9735	52,317
cell 20	3337	23,975		cell20	2451	53,149

Table A.3. cont'd FRET data

FRET 304+ α				**FRET 441+α**		
	Count	**Mean**			**Count**	**Mean**
cell1	1532	30,565		cell1	7751	45,191
cell2	1139	32,236		cell2	3319	55,298
cell3	697	43,487		cell3	2703	66,326
cell4	898	49,23		cell4	3750	63,309
cell5	246	48,522		cell5	5024	53,329
cell6	256	46.873		cell6	3374	59,799
cell7	1228	31,635		cell7	2719	63,646
cell8	432	49,669		cell8	4587	54,449
cell9	107	47,994		cell9	9913	53,211
cell10	1936	24,211		cell10	3680	60,892
cell11	680	41.694		cell11	7216	47,775
cell12	5131	29,728		cell12	5190	51,728
cell13	739	37,36		cell13	12606	46,619
cell14	2091	25,718		cell14	2163	72,4
cell15	1207	36.749		cell15	2710	66,52
cell16	916	34,575		cell16	2439	58,541
cell17	1319	37,269				
cell18	861	51.338				
cell19	4271	33,928				
cell20	196	46,478				

APPENDIX E

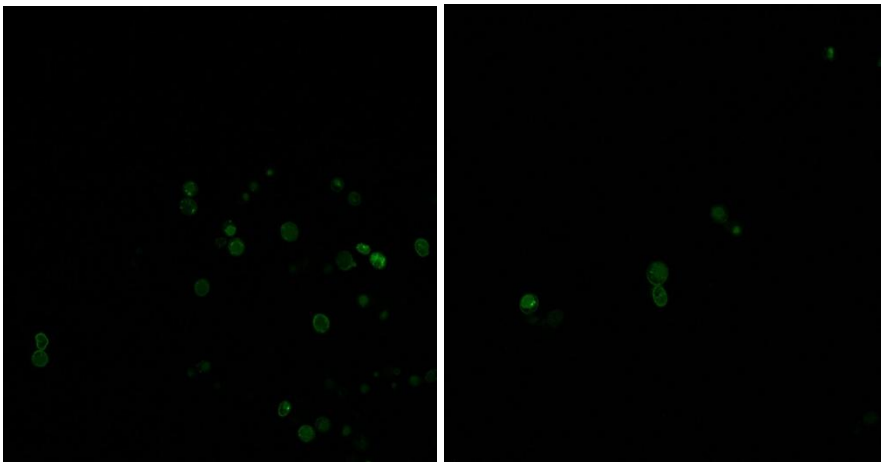
List of Drugs targeting the GPCRs (Veulens & Rodríguez, 2009).

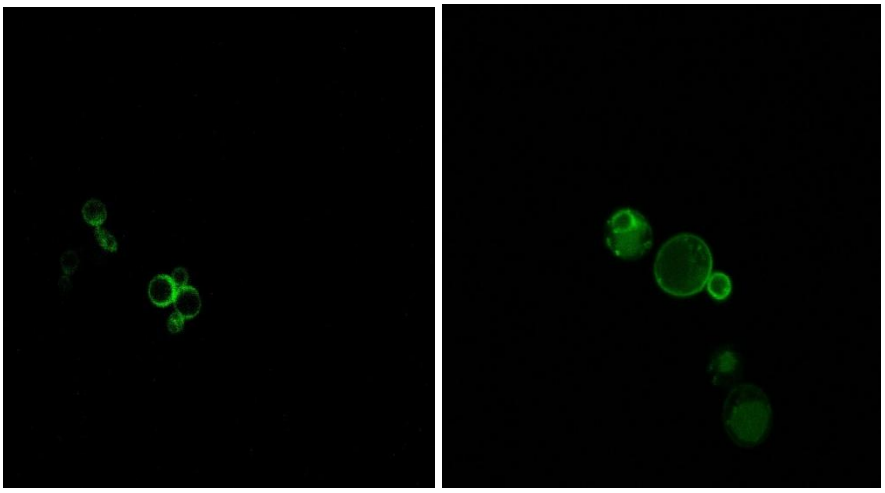
Trademark	**Generic name**	**Company**	**Disease**	**Target receptor**
Claritin	Loratadine	Schering-Plough	Allergies	H1 antagonist
Zyprexa	Olazapine	Eli Lilly Tatemoto	Schizophrenia	Mixed D2/D1/5-HT2
Cozaar	Losartan	Merk& Co	Hypertension	AT1 antagonist
Risperdal	Risperidone	Johnson& Johnson	Psychosis	Mixed D2/5-HT2
Leuplin/Lupron	Leuprolide	Takeda	Cancer	LH-RH agonist
Neurontin	Gabapentin	Pfizer	Neurogenicpain	GABA B agonist
Allegra/ Telfast	Fexofenadine	Aventis	Allergies	H1 antagonist
Imigran/ Imitex	Sumatriptan	GlaxoSmith Kline	Migrane	5HT1 agonist
Serevent	Salmaterol	GlaxoSmith Kline	Asthma	β2 agonist
Zantac	Ranitidine	GlaxoSmith Kline	Ulcers	H2 antagonist
Pepcidine	Famotidine	Merk& Co	Ulcers	H2 antagonist
Zofran	Ondansetron	GlaxoSmith Kline	Antiemetic	5-HT3 antagonist
Dovan	Valsartan	Novartis	Hypertension	AT1 antagonist
Duragesic	Fentanyl	Johnson& Johnson	Pain	Opioid agonist

APPENDIX F

Single Transfected Cell Images

EGFP 304 images





mCherry 304 images