Hes·so // VALAIS WALLIS
Haute Ecole Spécialisée de Suisse occidentale
Fachhochschule Westschweiz
University of Applied Sciences Western Switzerland

Domaine Sciences de l'ingénieur
Rte du Rawyl 47
CH- 1950 Sion 2
Tél. +41 27 606 85 11
Fax +41 27 606 85 75
info@hevs.ch

www.hevs.ch

Filière Technologies du vivant

Orientation Chimie analytique

Diplôme 2009

Fabien Rawyler

Analyse par imagerie FRET de l'auto-association de récepteurs membranaires en cellule vivante

Professeur JEAN—MANUEL SEGURA

Expert HORST VOGEL

Sion, le 18 août 2009

☐ FSI ☒ FTV	Année académique / *Studienjahr* **2008/09**	No TD / *Nr. DA* **ca/2009/92**
Mandant / *Auftraggeber* ☐ HES—SO Valais ☐ Industrie ☒ Etablissement partenaire **EPFL**	Etudiant / *Student* **Fabien Rawyler**	Lieu d'exécution / *Ausführungsort* ☐ HES—SO Valais ☐ Industrie ☒ Etablissement partenaire
Professeur / *Dozent* **Jean-Manuel Segura**	Expert / *Experte* *(données complètes)* **Prof. Horst Vogel** LCPPM | EPFL | 1015 Lausanne	
Travail confidentiel / *vertrauliche Arbeit* ☐ oui / ja [1] ☒ non / *nein*		

Titre / *Titel*

Analyse par imagerie FRET
de l'auto-association de récepteurs membranaires en cellule vivante

Description et Objectifs / *Beschreibung und Ziele*

Que ce soit dans le contexte du développement de nouveaux médicaments ou d'études fondamentales en biologie cellulaire, il existe un besoin croissant de développer de nouvelles techniques d'analyse permettant de mesurer l'association de protéines dans des conditions physiologiques, c'est-à-dire directement en cellules vivantes. Récemment , une nouvelle technique d'imagerie FRET (Fluorescence resonance energy transfer) combinée à un marquage fluorescent spécifique à l'aide d'une méthode enzymatique a permis d'obtenir des informations d'une précision quantitative inégalée sur l'association de protéines membranaires. Cette méthode n'ayant été appliquée qu'à l'étude d'un seul récepteur membranaire, il s'agira — dans le cadre de ce diplôme — d'étendre le champ de cette technique en l'appliquant à un, voire à plusieurs autres récepteurs membranaires et en comparant les résultats à ceux obtenus par des méthodes plus classiques.

Les objectifs de ce travail de diplôme sont les suivants :

— acquisition d'expertise en imagerie FRET cellulaire

— analyse de l'auto-association du récepteur beta2-adrénergique : 1) démonstration de l'association (ou non-association) du récepteur par l'existence (ou non-existence) d'un signal FRET; 2) analyse détaillée de cette association (degré d'oligomérisation; variation de l'association avec la concentration membranaire)

— éventuellement : extension à d'autres récepteurs membranaires (récepteurs olfactifs, etc.).

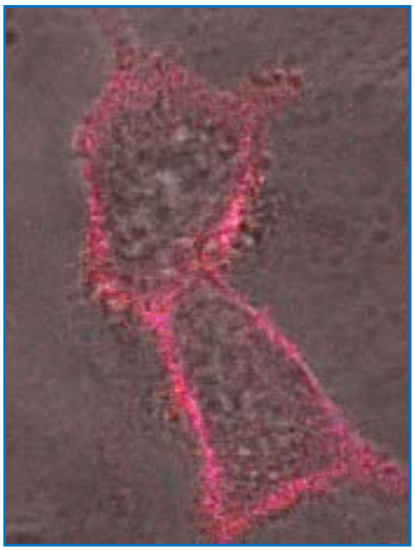
Signature ou visa / *Unterschrift oder Visum* Resp. de la filière *Leiter des Studieng.:* ..... [1] Etudiant / *Student*:	Délais / *Termine* Attribution du thème / *Ausgabe des Auftrags:* 27.04.2009 Remise du rapport / *Abgabe des Schlussberichts:* 19.08.2009 — 12h00 Exposition publique / *Ausstellung Diplomarbeiten:* 04.09.2009 Défense orale / *Mündliche Verfechtung:* semaines 36 ou 37 — selon programme

[1] *Par sa signature, l'étudiant-e s'engage à respecter strictement le caractère confidentiel du travail de diplôme qui lui est confié et des informations mises à sa disposition.*
Durch seine Unterschrift verpflichtet sich der Student, die Vertraulichkeit der Diplomarbeit und der dafür zur Verfügung gestellten Informationen zu wahren.

Rapport reçu le / *Schlussbericht erhalten am* ..... Visa du secrétariat / *Visum des Sekretariats* .....

Hes·so VALAIS WALLIS
Haute Ecole Spécialisée de Suisse occidentale
Fachhochschule Westschweiz
University of Applied Sciences Western Switzerland

Analyse par imagerie FRET de l'auto-association de récepteurs membranaires en cellule vivante



Diplômant Fabien Rawyler

Objectif du projet

Etudier par l'imagerie FRET le récepteur membranaire β_2-adrénergique exprimé dans des cellules vivantes (HEK 293) au vu de déterminer son auto-association. Les récepteurs sont marqués par réaction enzymatique avec deux fluorophores nommés Cy3 et Cy5 à différents rapports.

Méthodes | Expériences | Résultats

A partir du FRET apparent mesuré, le degré d'oligomérisation du récepteur β_2-adrénergique est de 4.1 ± 0.6 avec une distance de 64.8 ± 1.8 Å entre les récepteurs. Le récepteur β_2-adrénergique n'est pas sous forme de dimère.
Dans le domaine d'expression de récepteurs analysé, le FRET apparent n'est pas dépendant du niveau d'expression du récepteur. La déplétion du cholestérol de la membrane cellulaire ne montre pas de décroissance du signal du FRET apparent. Ceci ne permet dès lors pas de prouver la présence de microdomaines à sa surface.
Il y a donc une interaction protéine-protéine qui fait que le récepteur β_2-adrénergique est présent sous forme d'oligomère de structure tétramérique dans la membrane cellulaire au niveau d'expression étudié.

Objectives

Auto-association of the β_2-adrenergic receptor expressed in life cells (HEK 293) was studied by FRET imaging. Therefore, receptors are labelled by enzymatic reaction with two fluorophores, namely Cy3 and Cy5, in different ratios.

Methods / Experiences / Results

From the apparent FRET measured, the oligomerization degree of β_2-adrenergic receptors is 4.1 ± 0.6, with a distance of 64.8 ± 1.8 Å between receptors. β_2-adrenergic receptor does not appear as a dimeric structure in the plasma membrane.
From the expression levels that were analyzed, apparent FRET did not appear to be dependent on the expression level. Depletion of membrane plasma cholesterol does not show a decrease of apparent FRET signal. The presence of microdomains in the plasma membrane is not demonstrated.
A protein-protein interaction is proposed that make β_2-adrenergic receptors appear as oligomer with a tetrameric structure in the plasma membrane for analyzed expression level.

Travail de diplôme
| édition 2009 |

Filière
Technologies du vivant

Domaine d'application
Chimie analytique

Professeur responsable
Prof. Jean-Manuel Segura
jmanuel.segura@hevs.ch

Partenaire
Prof. Horst Vogel
Laboratoire de Chimie Physique des Polymères et Membranes (LCPPM)
EPFL

HES-SO Valais
Route du Rawyl 47
1950 Sion

Tél. 027 606 85 11
URL www.hevs.ch

Contents

1. Abbrevations

7TM	Seven transmembrane
A	FRET Acceptor
AcRe	Acceptors Receptors
ACP	Acyl carrier protein
BRET	Bioluminescence Resonance Energy Transfert
CoA	Coenzyme A
Cy3	Cyanine fluorescent dye
Cy5	Cyanine fluorescent dye
D	FRET Donor
DA	Ratio donor/acceptor
DMEM	Dulbecco's modified Eagle medium
DNA	Desoxyribo nucleid acid
E	FRET efficiency
$E_{app,se}$	FRET efficiency for sensitized acceptor emission
FBS	Fetal bovine serum
FR	FRET ratio
FRET	Fluorescence or Förster Resonance Energy Transfert
GPCR	G protein-coupled receptor
HEK	Human embryonic kidney
MBCD	Methyl-β-cyclodextrin
n	Number of units in an oligomer
PBS	Phosphate-buffered saline
r	Intermolecular distance
X_D	Donor mole fraction

2. Introduction

The First target in this diploma thesis was an introduction to cell biology and the basic manipulation of live cells, like cell culture maintenance, transfection or labelling.
The Second objective was the comprehension of Förster Resonance Energy Transfer (FRET) imaging in confocal microscopy and its application to a G-protein-coupled receptor (GPCR), the β_2-adrenergic receptor, to determine the degree of oligomerization.

2.1. Förster Resonance Energy Transfer

Förster resonance energy transfer (FRET) is an electrodynamic phenomenon that can be explained using classical physics. FRET occurs between a donor molecule (D) in the excited state by a laser and an acceptor molecule (A) in the ground state. Donor emit at shorter wavelength that overlap with the absorption spectrum of the acceptor. Energy transfer leads to the appearance of a photon and is the result of long-range dipole-dipole interactions between donor and acceptor. [Lakowicz, 2006]

FRET is determined by illuminating the donor and measuring emission from the acceptor. By using two different spectral variants of cyanine fluorescent dyes as the fluorophores, one can monitor the interaction of any two fluorophores and, when applied to the β_2-adrenegic receptor, the oligomerization of this receptor. [Alberts et al, 2002]

The explanation of this energy transfer is given by the Jablonski diagram (Figure 2.1). The diagram shows that the donor is excited (k_{ex}) by a photon into higher vibrational levels of the first singlet state S1 of the donor D*. Following vibrational relaxation to the lowest vibrational state of D*, the excitation energy is lost spontaneously by one of the following conversions: (k_{isc}) intersystem crossing to the triplet state, (k_f) radiative decay to the ground state, or the most important constant: (k_T) long-range resonance dipole energy transfer to an acceptor molecule close to the excited donor molecule. [Wieb Van Der Meer, 1994]

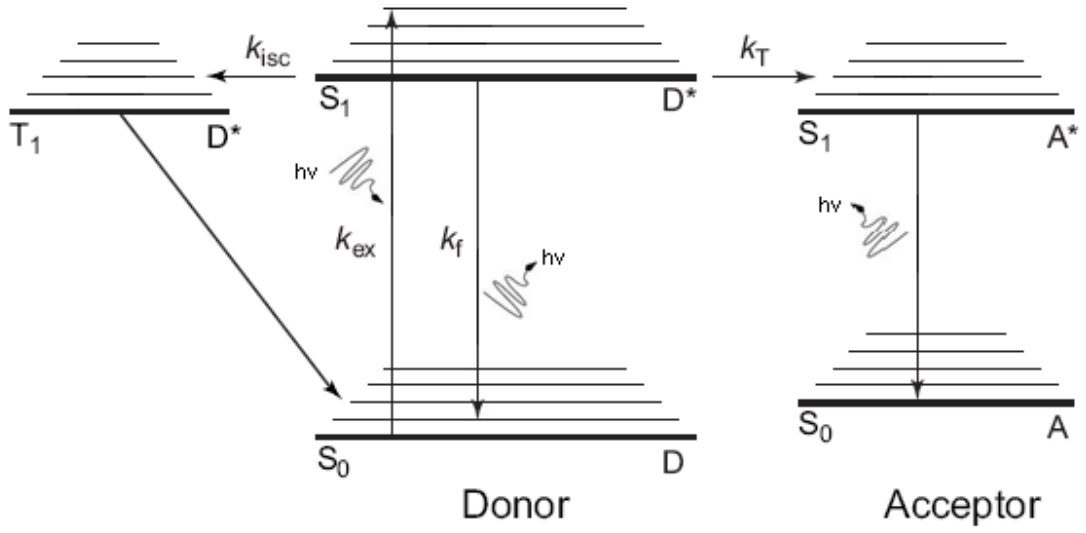


Figure 2.1: Jablonski diagram showing the transitions between donor and acceptor

Förster [Förster, 1948] has deduced an equation from the transfer signal to measure the distance between donor and acceptor (Equ. 2.1).

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

[Equ. 2.1]

Where:

E: efficiency of energy transfer
r: intermolecular distance
R_0: distance between donor-acceptor pair

The consequence of the sixth power of $\frac{r}{R_0}$ in Equ. 2.1 is that an increase in the distance between acceptor and donor from r = R_0 to r = $2R_0$ leads to a decrease in E from 50% to 1.5%.

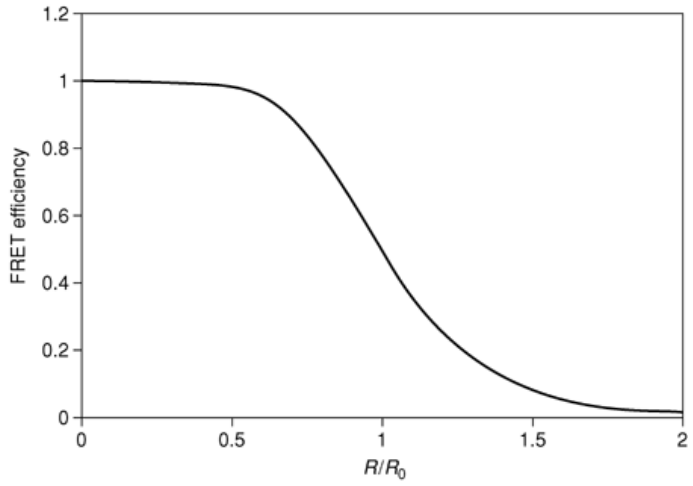


Figure 2.2: dependence of FRET efficiency to distance r [Meyer, 2005]

Another equation to describe the efficiency of energy transfer for a fixed distance between donor and acceptor is Equ. 2.2.

$$E = \left(\frac{F_{AD}}{F_A} - 1\right)\left(\frac{\varepsilon_A}{\varepsilon_D}\right)$$

[Equ. 2.2]

Where:

E: efficiency of energy transfer
F_{AD}: fluorescence intensity of the acceptor in the presence of the donor
F_A: fluorescence intensity of the acceptor only
ε_A: molar extinction coefficient of the acceptor
ε_D: molar extinction coefficient of the donor

In practice, Equ. 2.2 must be corrected for biological systems, where the distance r is not constant (Equ. 2.3).

$$E_{app} = \left(\frac{F_{AD}}{F_A} - 1\right)\left(\frac{\varepsilon_A}{\varepsilon_D}\right)\left(\frac{1}{X_a}\right)$$

[Equ. 2.3]

Where:

X_a: fraction of acceptor in complex with the donor

Finally, the FR (FRET ratio) can be calculated with a procedure on the IGOR Pro software (Version 6.02, Wavemetrics Inc.) developed by Meyer [Meyer, 2005] and adapted by J-B Perez, (Equ. 2.4).

$$\boldsymbol{FR = \frac{F_{AD}}{F_A} = \frac{Ff - S_1 \times Df}{S_4 \times Af}}$$

[Equ. 2.4]

Where :

Ff : signal from a donor-acceptor sample using FRET channel
Df : signal from a donor-acceptor sample using donor channel
Af : signal from a donor-acceptor sample using acceptor channel
S1, S4 : cross-talks

2.2. **Fluorophores**

A fluorophore is a functional group in a molecule that absorbs energy of a specific wavelength and re-emit energy at a higher wavelength. The amount and wavelength of the emitted energy depend on both the fluorophore and the chemical environment. [Lakowicz, 2006]
Different pairs of fluorophores can be used for measuring the FRET signal, like CFP/YFP, CF/TR or Alexa488/Cy3. [Pawley, 2006]
For this diploma thesis, the two fluorophores used were Cy3 and Cy5. They are water-soluble fluorescent dyes of the cyanine family. Cy3 is used as a donor and Cy5 as acceptor. [Pawley, 2006]

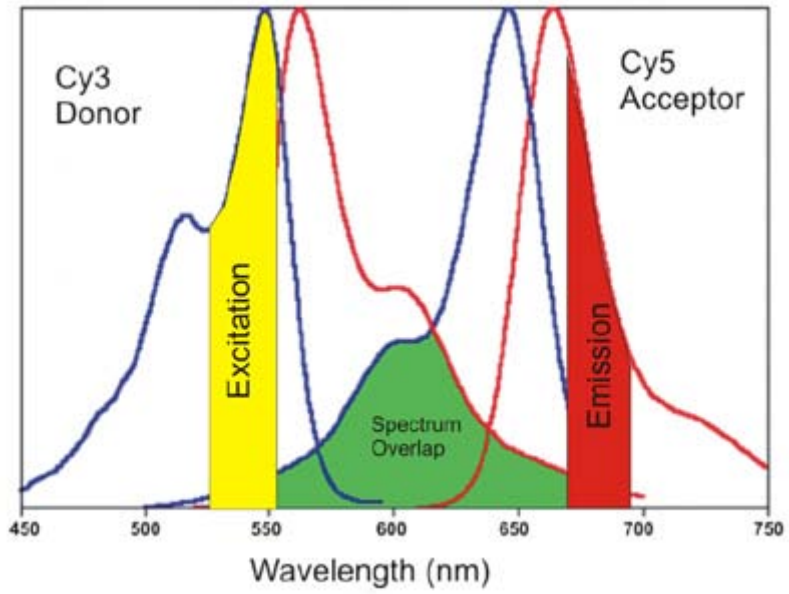


Figure 2.3: Excitation (blue line) and emission (red line) spectrum for Cy3 and Cy5 [Held, 2005]

Two key conditions required for FRET are: (i) spectral overlap between the emission of the donor molecule and the excitation of the acceptor and, (ii) close proximity between donor and acceptor (less than 100 Å). [Milligan, 2004]

For Cy3/Cy5, the two conditions are fulfilled. The spectral overlap is present as presented in Figure 2.3, and the distance between the donor and acceptor (R_0) is 50 Å. [Lakowicz, 2006]

2.3. Biological and pharmaceutical applications

FRET is a powerful technique for studying molecular interactions inside living cells with a very spatial (Ångstrom) and temporal (nanosecond) resolution, distance range, sensitivity, and a broad range of biological applications. [Sekar and Periasamy, 2003]

One application in biology is the onset and termination of Ca^{2+} signalling in cells, like the cytoplasm, nucleus, or endoplasmic reticulum, which can be observed by measuring of donor/acceptor intensities. [Truong et al., 2001]

FRET is used in molecular biology sees too an application of FRET technique for membrane fusion assays. In the lipid-mixing assay, membranes labelled with donor and acceptor lipid probes are mixed with unlabelled membranes. The FRET signal starts to decrease when the fusion between labelled and unlabelled membranes begins. [Sekar and Periasamy, 2003]

In bioanalysis, FRET immunoassays is an alternative to ELISA or others techniques using antigens and antibodies. [Miller, 2005]

Finally, the last application mentioned concerns carbohydrates. The FRET technique has permitted to define the flexibility and conformation of N-linked glycans. [Rice, 2001]

In this diploma thesis, we are interested by G-protein-coupled receptors. distance between two receptors is measured by using the FRET technique.

2.4. G-protein-coupled receptors

G-protein-coupled receptors (GPCRs) represent one of the largest and most diverse groups of proteins encoded by the genome in mammals.
Nearly 800 different human genes encode receptors for various extracellular ligands, including hormones, neurotransmitters and sensory stimuli. Moreover, these receptors are the targets of 30% of all pharmaceuticals on the market. [Oldham and Hamm, 2008]
All GPCRs have seven transmembrane-spanning (7TM) α-helices, an extracellular N terminus, an intracellular C terminus and three interhelical loops on each side of the membrane.
Based on phylogenetic analysis, human GPCRs can be classified into five main families of receptors: rhodopsin, secretin, glutamate adhesion and frizzled-taste-2. [Oldham and Hamm, 2008]
Family A (rhodopsin) is by far the largest group, and includes the receptors for light (rhodopsin), adrenaline (adrenergic receptors) and, most other 7TM receptor types, including the olfactory receptor subgroup. [Pierce et al., 2002]

For this diploma thesis, the receptor of interest is the β_2-adrenergic receptor.
It is part of a family of rhodopsin receptors which is a target for catecholamines, especially noradrenaline and adrenaline.
In 2007, the β_2-adrenergic receptor has been crystallized in a monomeric state and the structure was determined by high-brilliance microcrystallography. [Rasmussen et al., 2007]
However, it is unclear if this monomeric state represents a true physiological state or if the oligomeric state has been disrupted by purification or crystallization. [Dalrymple et al., 2008]

The β_2-adrenergic receptor can be activated with an agonist molecule like showed in Figure 2.4.

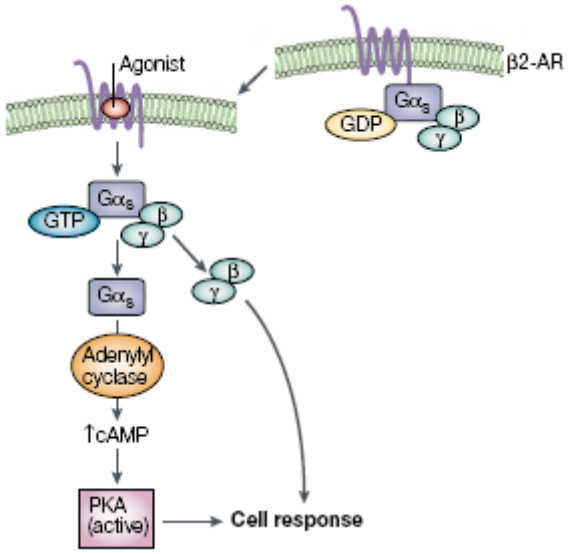


Figure 2.4 : In the absence of agonist, the β_2-adrenergic receptor are in the low –affinity strate. After agonist binding, a transient high-affinity complex of agonist, activated receptor and G protein is formed. GDP released frome G protein and is replaced by GTP. This leads to dissociation of the G-protein complex into a-subunits ans bg dimers, wich both activte several effectors. [Pierce et al., 2002]

This particular receptor (β_2-adrenergic receptor) is one of the most studied GPCRs with respect to agonists and antagonists [Breit et al., 2004].

3. Materials and Methods

3.1. Materials

Biological and chemical materials used :

- ACP-β_2-adrenergic DNA gift from Karen L. Martinez, Nano-Science center, Copenhagen
- CoA-Cy3, CoA-Cy5, AcpS gift from Ruud Hovius, Institute of Chemical Sciences and Engineering, Lausanne
- DMEM/F12 + GlutaMax, Gibco 31331, Invitrogen
- Effectene®, Qiagen, Cat N° 301425
- Fetal Bovine Serum, Sigma F7524
- HEK 293T/17, ATCC CRL-11268, LGC
- G418 sulfate, Gibco 11811023, Invitrogen
- Isoproterenol, Sigma I5627
- Methyl-β-cyclodextrin, Sigma C4555
- $MgCl_2 \cdot 6H_2O$ > 99.0%, Fluka 63065
- PBS 10x pH 7.2, Gibco 70013, Invitrogen
- Trypsin-EDTA solution (1x), Sigma T3924
- Water, NANOpur, Barnstead

Materials used :

- 8-well plate borosilicate,Nunc
- Centrifuge, Eppendorf 5415 D
- Centrifuge, Eppendorf 5810 R
- Confocal microscope LSM 510, Zeiss
- Glass coverslips 0.17 mm thick X 25 mm diameter, Assistant
- Glass coverslips 24 X 50 mm, Menzel-Gläser
- Incubator Hera cell 150, Hereaeus
- Microscope Axiovert 25, Zeiss
- Tissue culture flasks 25 cm^2, TPP 90025

3.2. Methods

3.2.1 Cell culture maintenance

HEK 293

Adherent HEK 293 were grown in DMEM/F12 supplemented with 10% FBS and maintained at 37 °C. Cells were sub-cultivated twice a week at ratios between 1:10 and 1:20.

Stable HEK 293

Stable HEK 293 cell lines with ACP-β_2-adrenergic receptor were maintained by selection with 200 µg/ml G418 in DMEM/F12 supplemented with 10% FBS.

Cell culture in 8-well

HEK 293

For the preparation of 8-well plates, 50 µl of cells in DMEM/F12 and 10% FBS were added in each well. 50 µl medium DMEM/F12 are supplemented with 10% FBS and finally 100 µl of DMEM/F12 was added. The final concentration of FBS was 5% and the concentration of cells was $1*10^5$/ml.
8-well plates were kept at 37 °C. Cells were transfected after 24h. [Meyer, 2005]

Stable HEK 293

For the preparation of 8-well plates, 100 µl of cells in DMEM/F12 with 200 µg/ml G418 and 10% FBS were added in each well, and finally 100 µl of DMEM/F12 was added. The final concentration of FBS was 5% and the concentration of cells was $1*10^5$/ml.
8-well plates were kept at 37 °C. Analysis by confocal microscopy was made after 48 h.

3.2.2 Transfection

Two different methods for transfection were used. The objective was to have a fast method with a good yield, at a minimum cost.

3.2.2.1 Calcium-phosphate method

Transfection by using the calcium-phosphate method for 8 well plates: [Jordan et al., 1996]

4.5 µl plasmid DNA (0.1 µg/µl) in 200 µl phosphate buffer (140mM NaCl, 1.4mM Na_2HPO_4, 50mM HEPES, pH 7) were added with 200 µl 250mM calcium buffer. Cells were transfected after exactly 1 minute by adding 50 µl were tranvased in each well and there after incubated at 37 °C. 4 h later, the medium was changed to 200 µl DMEM/F12 supplemented with 5% FBS at 37 °C. Analysis by confocal microscopy was made 48 h later.

3.2.2.2 Effectene® method

Transfection by using Effectene® in reagent for 8 well plates based on Qiagen method.
3.2 µl DNA (0.1 µg/µl) were added in 160 µl Buffer EC. 2.56 µl Enhancer were added. The solution was vortexed for a few seconds. After 5 minutes, 3.2 µl Effectene® were added. Finally, 10 minutes later, 20 µl were transferred in each well then incubated at 37 °C. Analysis by confocal microscopy was made 48 h later.

After comparing the two methods, a choice was made in favour of Effectene® transfection. This method is more repeatable, faster and easier than calcium-phosphate transfection.

3.2.3 ACP labelling

Before labelling, cells were washed once with 100 µl 1X PBS. Labelling was performed in 1X PBS with 10mM MgCl2, 1μM AcpS and 5 μM CoA substrate. Total volume was 52 µl. For double labelling with Cy3 and Cy5, the substrates, CoA-Cy3 and CoA-Cy5, were mixed beforehand at desired ratios to a total concentration of 5 μM. The labelling was performed at ambient temperature for 40 minutes. After incubation the cells were washed three times with 1X PBS and the cells were maintained with 200 µl 1X PBS. [Meyer, 2005].

3.2.4 Internalization assay

The cells were labelled as described in point 3.2.3. For induction of receptor endocytosis, cells were stimulated for 30 min with 1 µM isoproterenol at 37°C. [Breit and al., 2004]

3.2.5 *Cholesterol extraction*

After labelling of cells with Cy3 and Cy5, cholesterol was extracted with 2% methyl-β-cyclodextrin (MBCD) for 45 minutes at room temperature.

3.2.6 Confocal microscope

Laser-scanning confocal micrographs were recorded on a LSM 510 microscope (Zeiss) with a 63X (1.2 NA, W Korr, Zeiss) objective by using 543 nm and 633 nm HeNe lasers. Detection and distinction of fluorescence signals was achieved by appropriate filter sets using a multitracking mode (See Table 3.1).

Table 3.1 Multitracking parameters

Channel	Excitation Line	Dichroic Mirror (HFT)	Beam Splitter (NFT)	Emission Filter
Donor	543 nm	UV/488/543/633	635 Vis	BP 560-615
Acceptor	633 nm	UV/488/543/633	635 Vis	LP 650
FRET	543 nm	UV/488/543/633	635 Vis	LP 650

The donor and FRET images were taken at same time, because they are excited at the same wavelength. laser Intensity at 543 nm was between 126 to 700 nW (70%) and at 633 nm between 84 to 767 nW (10%).
For experiments, laser power was adjusted according to the level of receptor expression at the cell surface. Channel intensity was generally between 1’000 and 3’000 (units?) on plasma membrane.
Another important parameter in confocal microscopy is pinhole aperture size. In our experiments, it was 2.96 Airy units. This parameter defined the axial resolution (z-axis). [Pawley, 2006]
For more information about confocal microscopy methodology and practices, refer to the work of Bruno Meyer. [Meyer, 2005]

4. Results

4.1. Cross-talk calculations

To calculate the FRET ratio exactly, cross-talk is a very important parameter. Cross-talk depends on many parameters of signal acquisition, like laser power, settings and intensity.

First assay was a direct measure. a solution of Cy3 (or Cy5) in PBS was measured with the 3 channels. The problem was that donor and acceptor channel had a high intensity and that the FRET channel had a low intensity. Cross-talks are changing according to intensity. This way to calculate cross-talks S1 and S4 was not one.

The second method to try was using a solution of Cy3 (or Cy5) in PBS and with a change of laser power.

For the first step, a solution of Cy3 30 µM in PBS (or Cy5 14.8 µM) was added on a coverslip until the intensity of the histogram on the Zeiss software was a Gaussian. Then different images were made with different powers on all intensity ranges.
The second step was to make a dilution for that FRET channel and donor (or acceptor) channel could be measured with the same solution. This step enables one to know the dilution factors between the different assays.
The Final step was the same as the first step, but the solution was more diluted for the donor (or acceptor) channel.

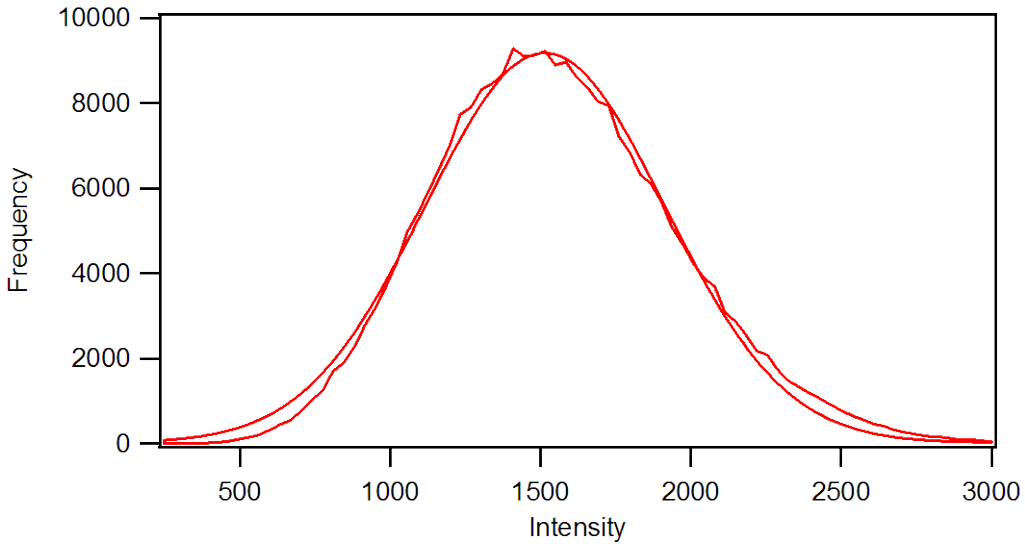


Figure 4.1: Histogram for acceptor channel with Cy5

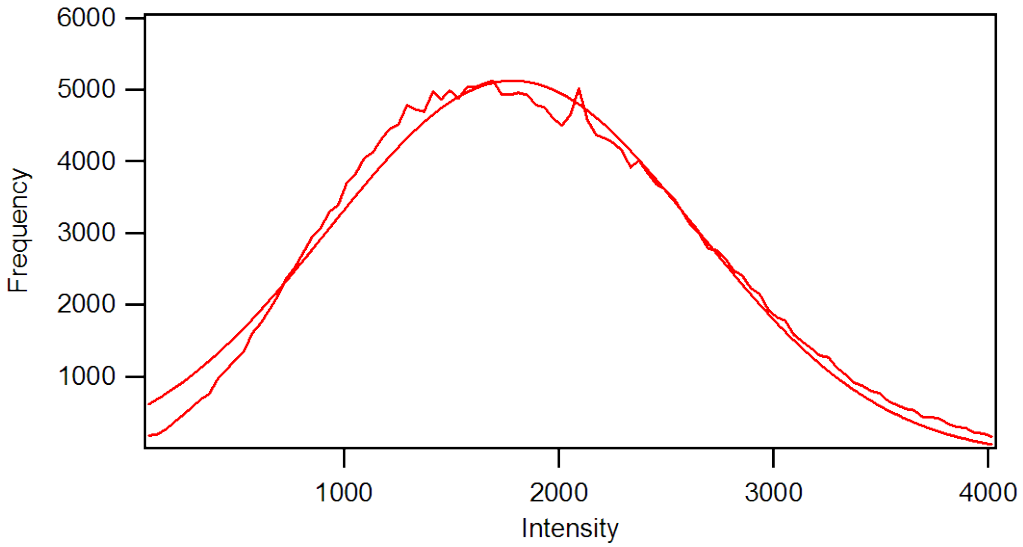


Figure 4.2: Histogram for FRET channel with Cy5

Based on histograms (Figure 4.1 and Figure 4.2), a graph was made for calibration of intensity as a function of laser power. The laser power response was linear with intensity for both acceptor channel and donor channel (Figure 4.3 and Figure 4.4).

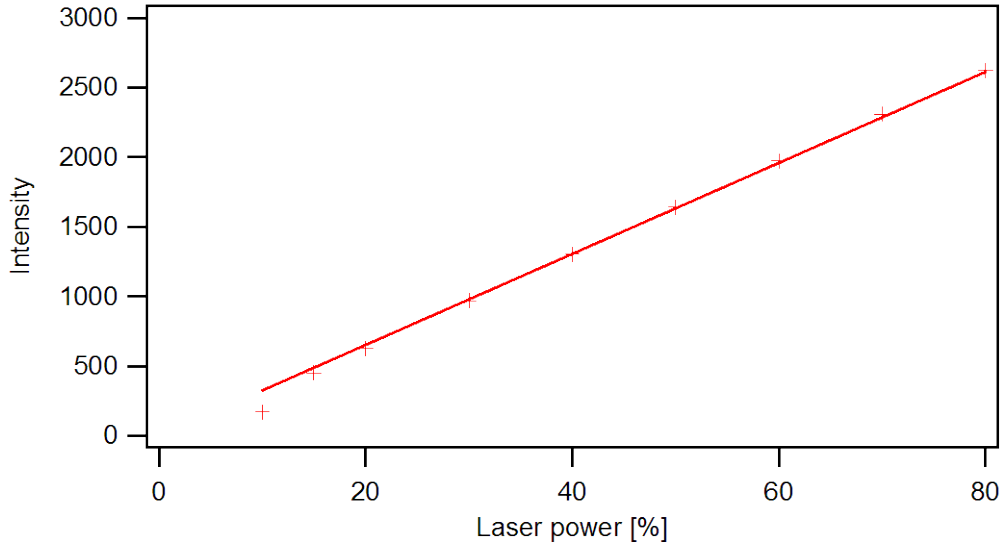


Figure 4.3: Intensity as a function of laser power for donor channel with Cy3. Slope 32.6 ± 0.4 and R^2 = 0.9990

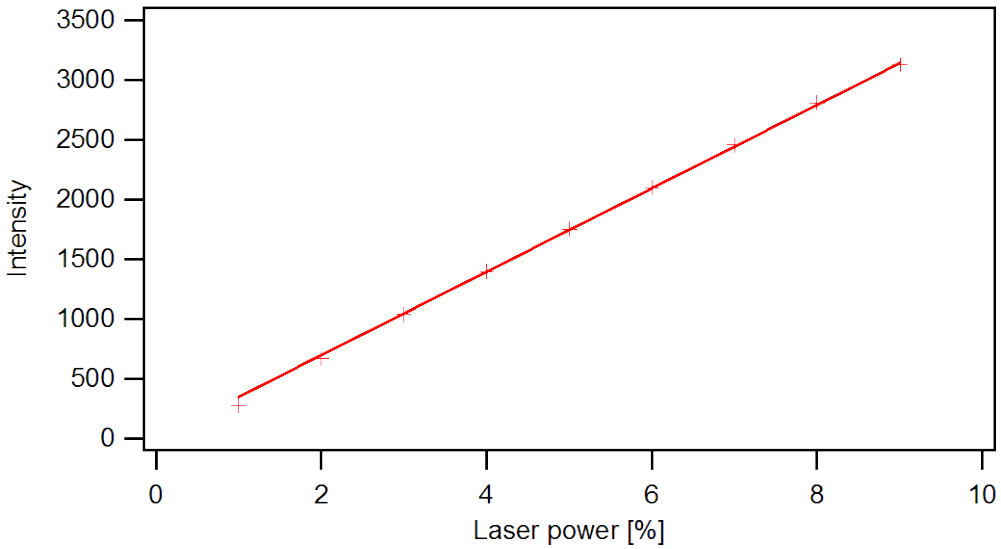


Figure 4.4: Intensity as a function of laser power for acceptor channel with Cy5. Slope 349 ± 2 and R^2 = 0.9990

However, no linearity has been observed in the FRET channel (Figure 4.5).

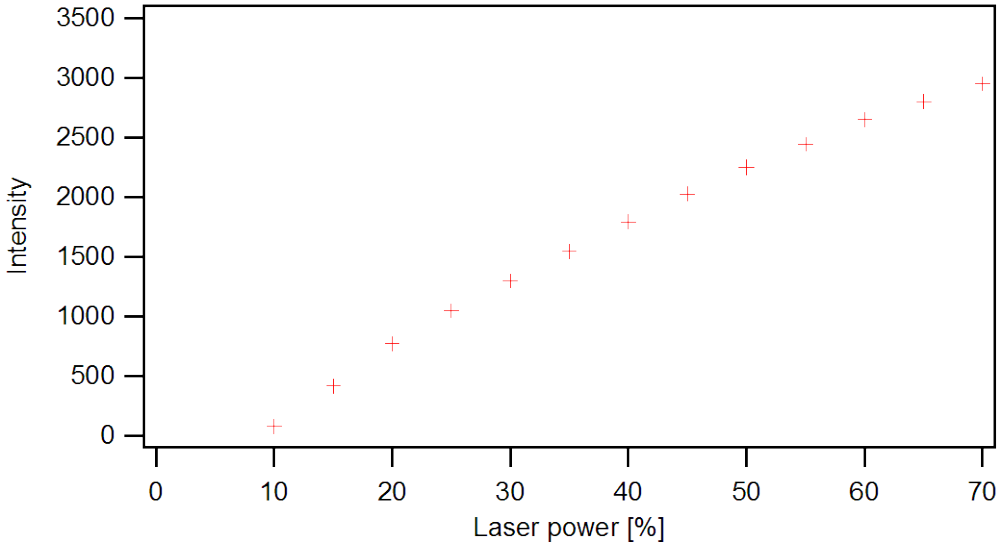


Figure 4.5: Intensity in function of laser power for FRET channel with Cy3

To solve this problem, a double calibration curve was made in function of intensity.

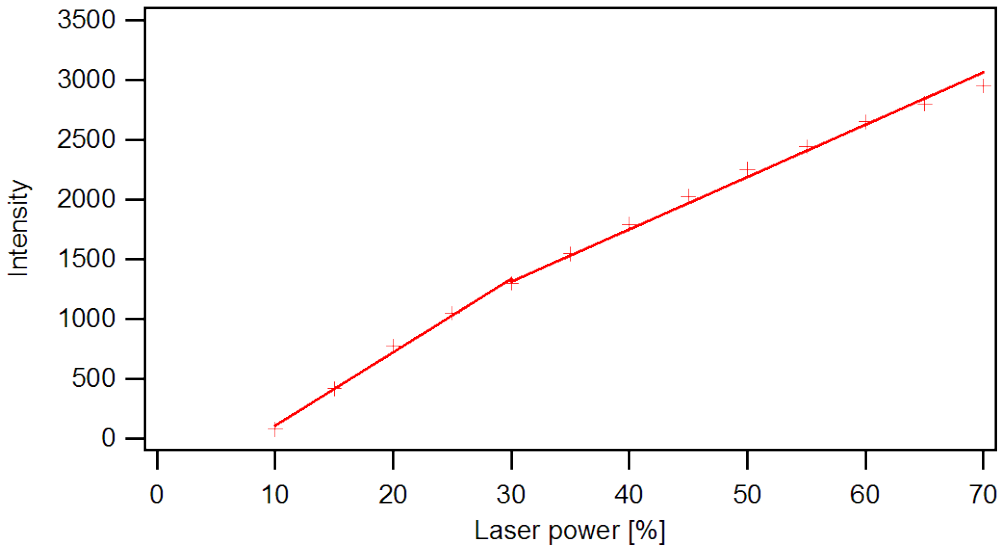


Figure 4.6: Intensity as a function of laser power for FRET channel with application of two calibration curves with Cy3. High curve: slope 43.7 ± 0.4 and R^2 = 0.996, low curve: slope 61 ± 3, y-intercept 508 ± 56 and R^2 = 0.997

Using two calibration curves (Figure 4.6), it is possible to obtain an all intensities range for the FRET channel. For donor and acceptor, these problems were not present.

From the different calibration curves, we can calculate cross-talks S1 (Equ. 4.1) and S4 (Equ. 4.2).

$$S1 = \frac{Int.Cy3\ (FRET\ channel)}{Int.Cy3\ (Donor\ channel)} \text{ and } S4 = \frac{Int.Cy5\ (FRET\ channel)}{Int.Cy5\ (Acceptor\ channel)}$$

[Equ. 4.1 and Equ. 4.2]

Error propagation calculation gives a maximal relative error of 11.9% for S1 and 2.4% for S4. [Skoog et al., 2007]

From S1 and S4, one can calculate the FR mean relative error with the following parameters (Table 4.1).

Table 4.1 Parameters to the calculation of FR mean relative error

	Value	Δ value
Acceptor channel [Intensity]	1000	50
FRET channel [Intensity]	1000	90
Donor channel [Intensity]	1000	50
S1	0.126	0.002
S4	0.400	0.004

The propagation error in FR for the last parameters (Table 4.1), FR was 2.19 and mean error was 0.11. In percentages, the mean relative error was 5.2%.

Still, the main FR error comes from intensity on the plasma membrane. In fact, depending on the threshold applied, results can change from 10 to 20%. In our experiment, threshold was increased to keep the highest plasma membrane intensity until the FR histogram looks like a normal distribution (see Figure 4.11).

For FR calculation, Equ. 2.4 was modified as a function of FRET channel intensity. If intensity was under the crossing between the high curve and low curve, Equ. 4.3 was applied.

$$FR = \frac{F - \left[D \times S1 \times \left(\frac{S1A2 \times \left(\frac{F}{S1A1}\right) + S1B2}{F}\right)\right]}{A \times \left[S4 \times \left(\frac{S4A2 \times \left(\frac{F}{S4A1}\right) + S4B2}{F}\right)\right]}$$

[Equ. 4.3]

Where:

S1A1: slope for high curve in FRET channel with Cy3
S1A2: slope for low curve in FRET channel with Cy3
S1B2: intercept for low curve in FRET channel with Cy3
S4A1: slope for high curve in FRET channel with Cy5
S4A2: slope for low curve in FRET channel with Cy5
S4B2: intercept for low curve in FRET channel with Cy5

4.2. Labelling of β_2-adrenergic receptors

ACP-β_2-adrenergic receptors were double labelled with Cy3 and Cy5.
Only extracellular receptors were labelled. Donor is shown by the left image, centre is acceptor and FRET right. The colours are artificial. Detectors give only an intensity.

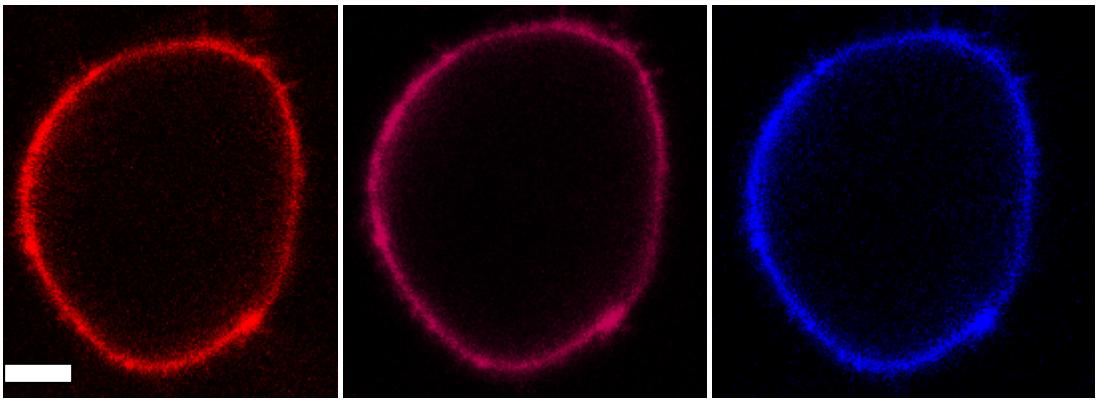


Figure 4.7: labelling of β_2-adrenergic receptors with Cy3 (left), Cy5 (centre). FRET signal is right. Donor ratio was DA 0.67/0.33. Scale bar: 5 µm.

Like demonstrated by Bruno Meyer [Meyer, 2005], for a receptor fused at its N-terminus with ACP and double labelled with CoA-Cy3 and CoA-Cy5, the Donor/Acceptor ratios in solution for labelling should correspond exactly to the real DA ratios obtained on the cells.

4.3. Internalization assay

A test to observe internalization of β_2-adrenergic receptors was effected according method 3.2.4. Endocytosis was initiated by isoproterenol. This assay permits to know if the labelling is specific for β_2-adrenergic receptor.

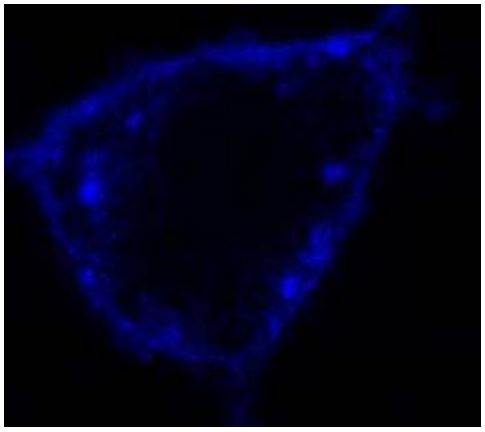


Figure 4.8: FRET canal image: cell in contact with 1 µM isoproterenol labelled with X_D 0.65

We can see that with isoproterenol (Figure 4.8), FRET signal is on the plasma membrane and inside the cell. This phenomenon is explained by endocytosis of the β_2-adrenergic receptor. This experiment confirms that β_2-adrenergic receptors are internalized in presence of isoproterenol and it is an indication for the proper functioning of our receptor.

4.4. Photobleaching

Photobleaching was recorded for the highest laser power used for measuring the FR. A degradation of donor or acceptor will give a mistaken value for FR.
Scan speed was set to 2.56 *µ*s (same parameters as for all images). 543 nm and 633 nm lasers were used for illuminating of, respectively, Cy3 (Figure 4.9) and Cy5 (Figure 4.10).

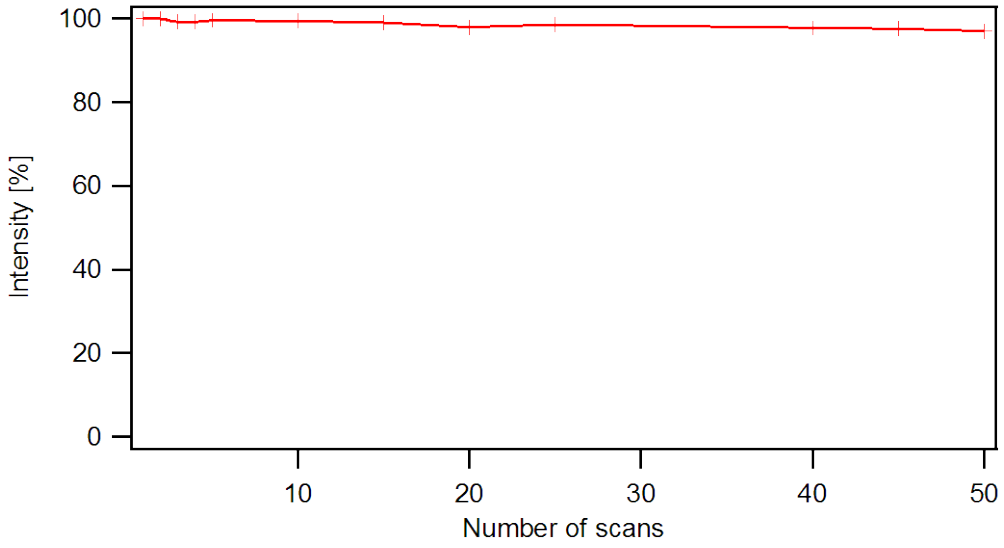


Figure 4.9: Measure of intensity for Cy3 as a function of number of scans at 543 nm and 70% laser power

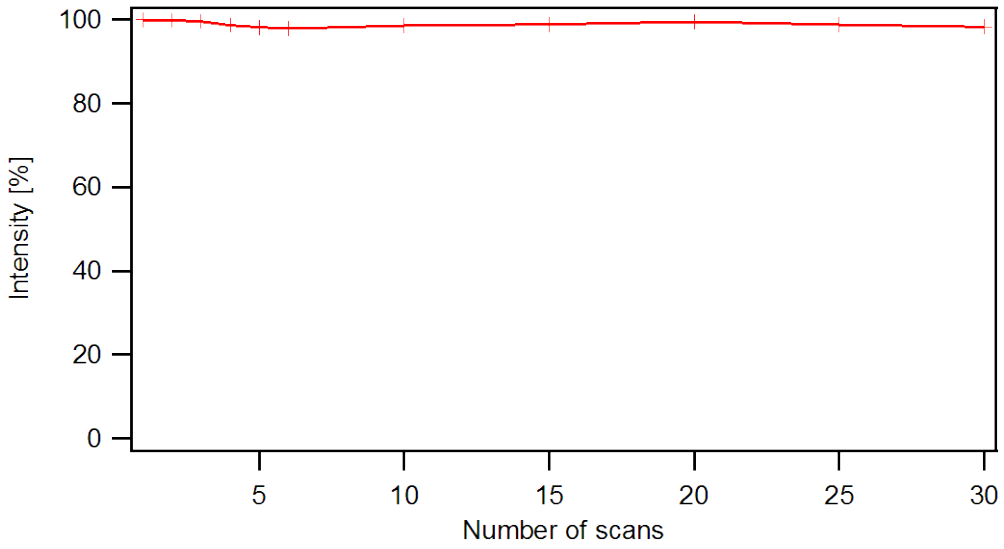


Figure 4.10: Measure of intensity for Cy5 as a function of number of scans at 633 nm and 10% laser power

After 30 scans at 543 nm, intensity decreases from 100 % to 98.3 %. At 633 nm and after 50 scans, intensity decreases from 100 % to 97.1 %.
Intensities for Cy3 and Cy5 did not decrease significantly with the number of scans for lasers power of 70% at 543 nm and 10% at 633 nm.
There is thus no photobleaching for the two highest lasers power used.

4.5. Oligomerization of β_2-adrenergic receptor

FR calculations have been performed using IGOR software. Methodology for the treatment of results was explained by Bruno Meyer. [Meyer, 2005]

Figure 4.11 shows FR on plasma membrane of two cells (left) and the FR value (right).

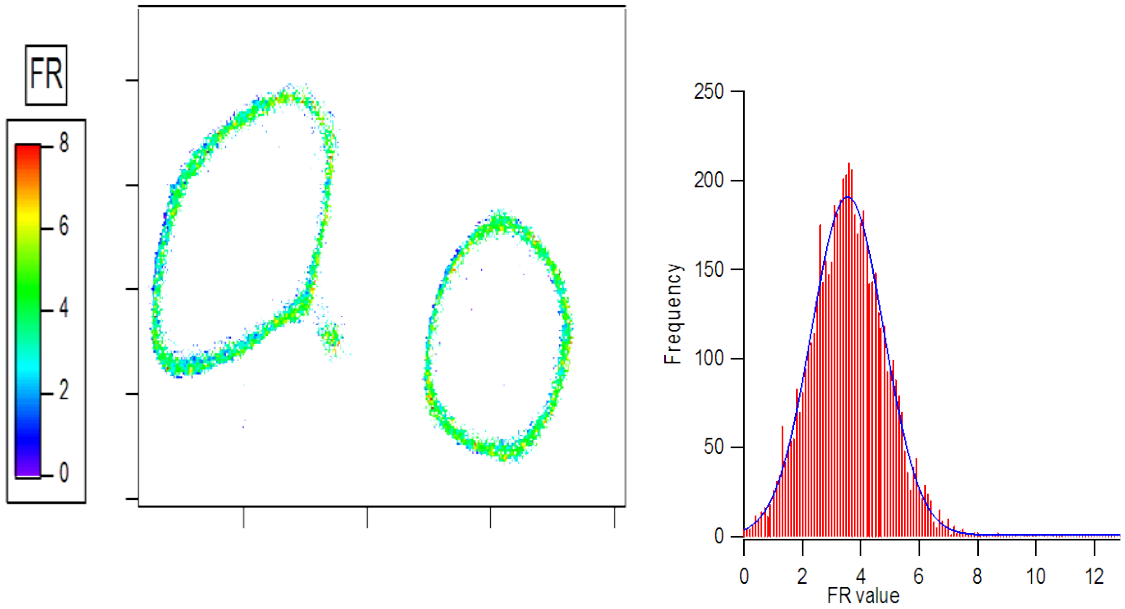


Figure. 4.11: FR image (left) and FR histogram (right) value for two cells with DA ratio 0.65/0.35.

To obtain FRET efficiency for sensitized acceptor emission ($E_{app,se}$) from FR, Equ. 4.4 has been proposed. [Erickson et al., 2001]

$$E_{app,se} = (FR - 1) \times \left(\frac{\varepsilon_A}{\varepsilon_D}\right)$$

[Equ. 4.4]

Oligomerization degree can be calculated with $E_{app,se}$ and X_D. From different theories [Adair and Engelman, 1994; Veatch and Stryer, 1977], Meyer has developed a model to calculate FRET efficiency (E) and number of units in an oligomer (n) with Equ. 4.5.

$$E_{app,se} = E\frac{x_D}{1 - x_D}(1 - x_D^{n-1})$$

[Equ. 4.5]

To apply this model, receptors must be completely labelled. This hypothesis is admitted to be fulfilled in our experiment.

Calculation of receptor density is not indicate with a concentration but calculated only with acceptors receptors labelled.
Like acceptor is proportional to X_D [Meyer, 2005], acceptors receptors (AcRe) is calculated by Equ. 4.6.

$$AcRe = X_D \frac{A\ Intensity}{Laser\ Power\ at\ 633\ nm\ [\%]}$$

[Equ. 4.6]

The ratio between acceptor intensity at the plasma membrane of each cell is given by IGOR software, and divided by the laser power in percentage, multiplied with the donor mole fraction.

The first hypothesis is that β_2-adrenergic receptors are expressed as a dimer, with n = 2. [Angers et al. (2002); Mercier et al. (2004)]
Number of acceptors receptors expressed at the cell surface is between 50 to 300.

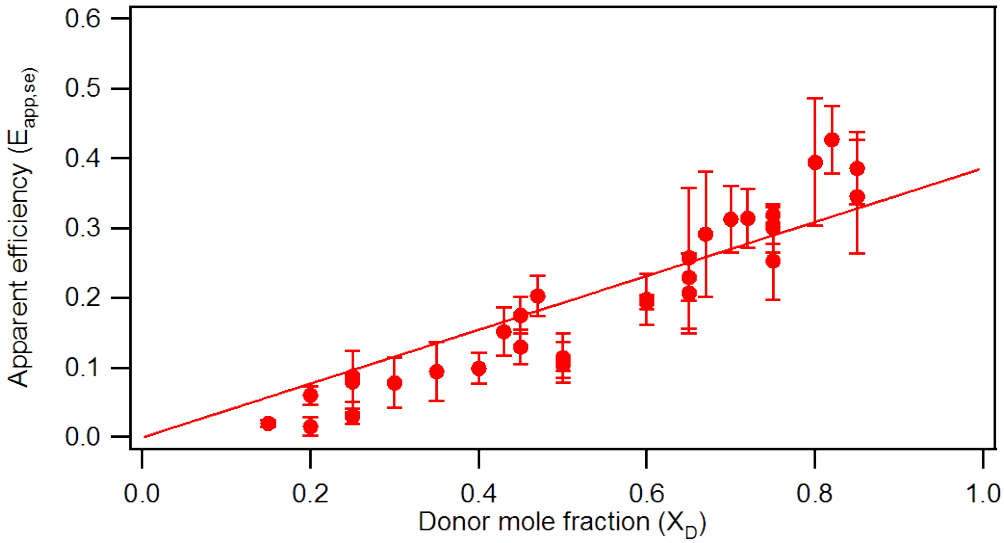


Figure 4.12: $E_{app,se}$ between β_2-adrenergic receptors at different donor mole fraction with n set to 2 between 50 to 300 acceptors receptors

With β_2-adrenergic receptors, n= 2 (Figure 4.12) in Equ 4.5, the true Efficiency, E= 0.39 ± 0.02. Each point is a mean (± SD) of 4 to 12 cells.
One can see that with $X_D < 0.5$, all points are under the linear curve, and with $X_D > 0.65$, points are above the linear curve.
There is clearly a deviation of the line and order 2 (n=2) does not correspond to an accurate fit.

The second case is with n not restricted.

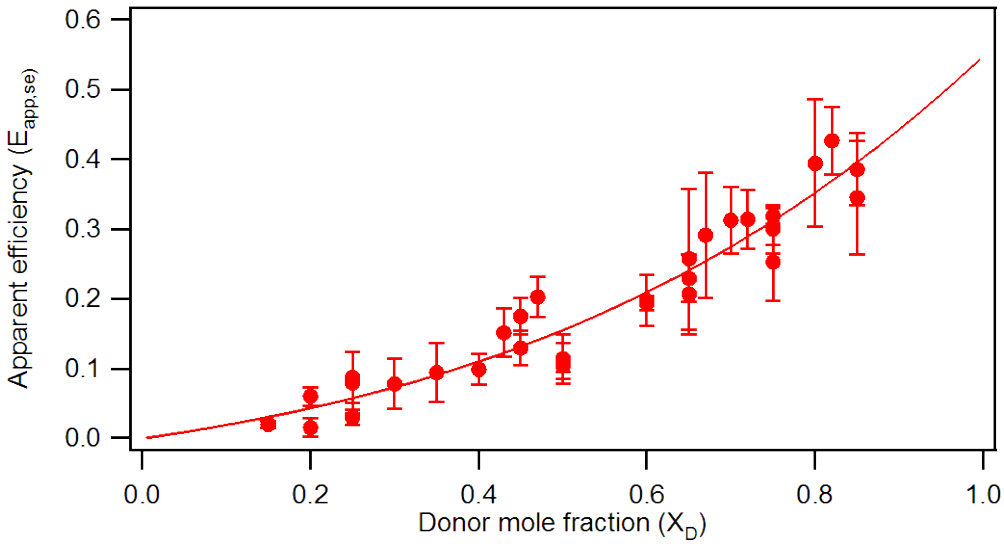


Figure 4.13: $E_{app,se}$ between β_2-adrenergic receptors at different donor mole fraction for AcRe between 50 to 300 acceptors receptors

After application of Equ 4.5 on Figure 4.13, the true Efficiency E= 0.18 ± 0.02 , and the oligomerization degree n= 4.1 ± 0.5. Each point is a mean (± SD) of 4 to 12 cells.
Thus the β_2-adrenergic receptor is present in the plasma membrane as an oligomer, probably as a tetramer.

With a restricted domain of acceptor receptors, range 150-200 AcRe, the oligomerization degree does not change and stays around 4.

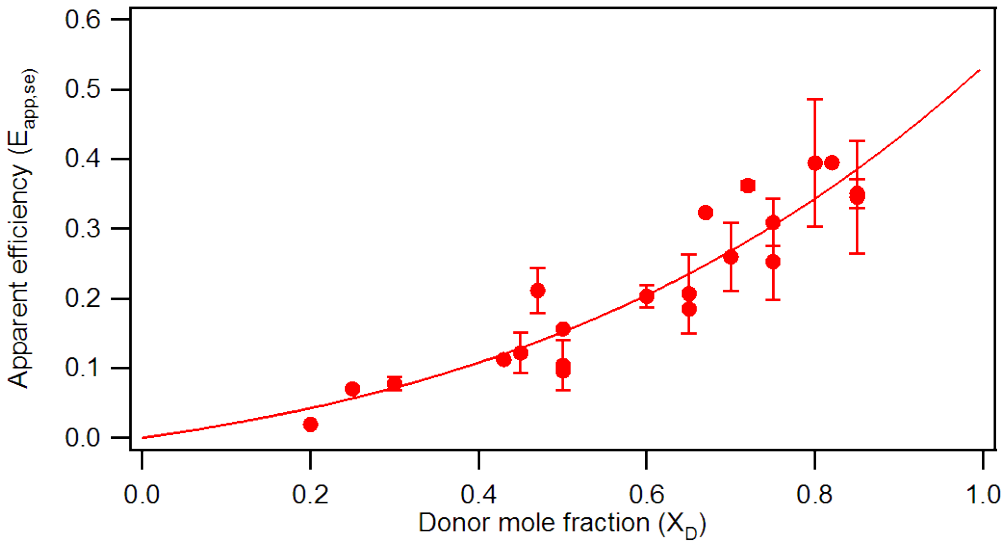


Figure 4.14: $E_{app,se}$ between β_2-adrenergic receptor at different donor mole fractions for AcRe between 150 and 200

After application of Equ 4.5 on Figure 4.14, the true Efficiency E= 0.17 ± 0.02 , and the oligomerization degree n= 4.1 ± 0.8. Each point is a mean (± SD) of 1 to 9 cells.

Assay was performed with a stable HEK 293 ACP-β_2-adrenergic receptor. The domain Range of AcRe is 130-450.

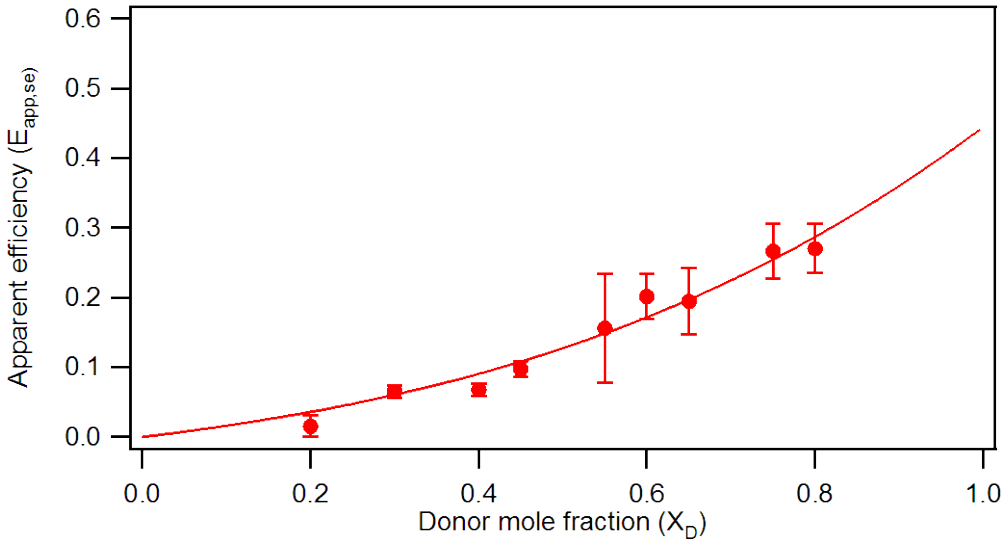


Figure 4.15: $E_{app,se}$ between β_2-adrenergic receptor at different donor mole fraction for stable HEK293

After application of Equ 4.5 on Figure 4.15, the true Efficiency E= 0.14 ± 0.02 , and the oligomerization degree n= 4.1 ± 0.7. Each point is a mean (± SD) of 7 to 11 cells.

We can also obtain the intermolecular distance (r) by Equ. 4.7.

$$r = \sqrt[6]{\frac{{r_0}^6 - E \times {r_0}^6}{E}}$$

[Equ. 4.7]

The distance between Cy3-Cy5 is 50 Å (R_0) [Kenworthy and Edidin, 1998], with E= 0.18 ± 0.02. In cases with a range between 50 and 300 AcRe, the calculated intermolecular distance is 64.74 ± 1.74 Å.

4.6. Dependence of FRET ratio on receptors density

To determine dependence of FRET to the density of receptors, all points with a DA ratio of 0.5/0.5 have been used, from experiments of three different days. Number of cells were N= 24.

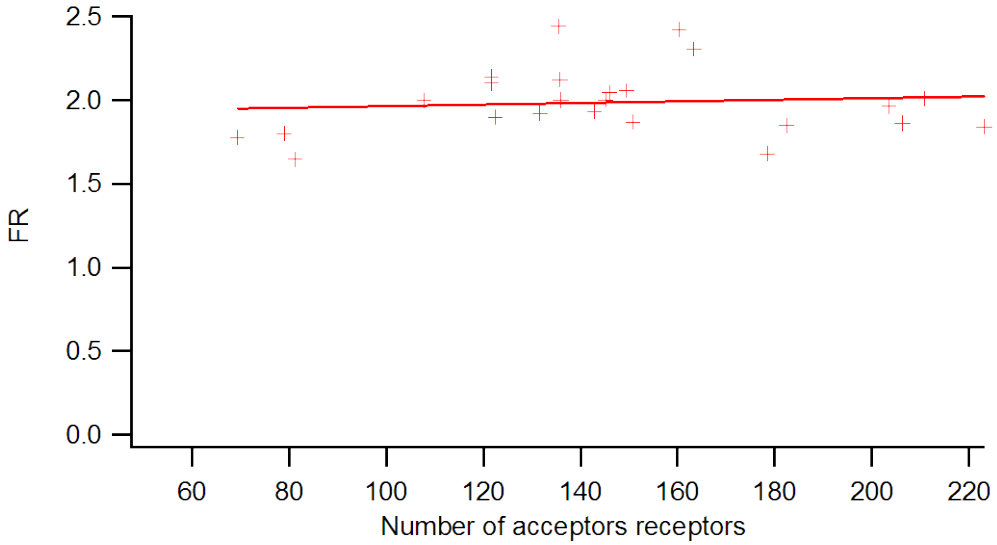


Figure 4.16: Variation of FR with the number of acceptors receptors for DA ratio 0.5/0.5

Figure 4.16 was fitted with a linear function. The intercept is 1.919 ± 0.159 and the slope 0.00047 ± 0.00105. Slope can be considered zero within the margin of error.

Same Figure is made for a DA ratio of 0.85/0.15 for three different days and N= 18:

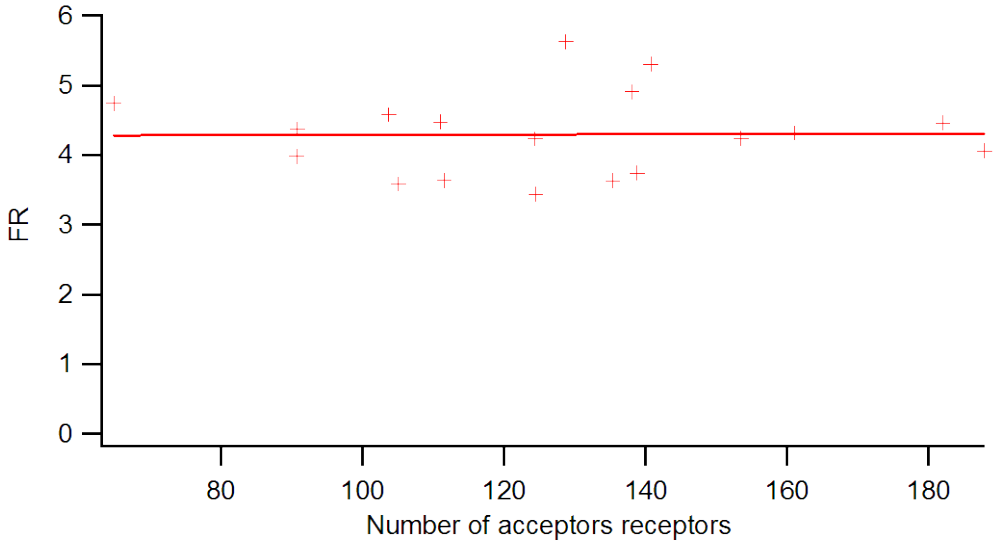


Figure 4.17: Variation of FR with the number of acceptors receptors for DA ratio 0.85/0.15

Figure 4.17 was fitted with a linear function. The intercept is 4.269 ± 0.618 and the slope is 0.00029 ± 0.00472.

Consequently, for the expression cells obtained, there is no dependence between FR and the number of receptors on a range of 4.

4.7. Cholesterol depletion

For cholesterol depletion, method 3.2.5 was used. Cells were labelled with a donor mole fraction of X_D= 0.63.

Two samples of cells are analyzed. One with 2% MBCD and another without MBCD like control (Figure 4.18).

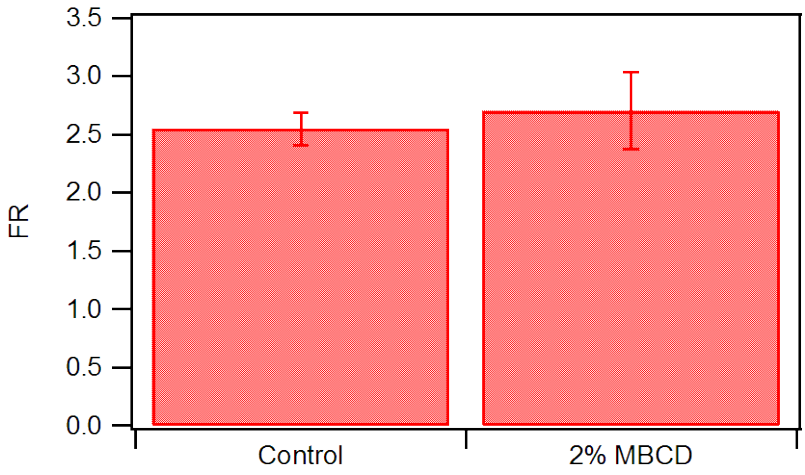


Figure. 4.18: Variation of FR by cholesterol extraction for a donor mole fraction of X_D=0.63 (± SD)

A T-Test is done to determine if segregation is present between control cells and cells treated with 2% MBCD.

Table 4.2: Cholesterol depletion results

	Sample	
	control	2% MBCD
cells number	11	7
average [FR]	2.55	2.7
standard deviation [FR]	0.14	0.33
S	0.231	
t	1.314	

For cholesterol depletion results, t obtained is 1.314. (Table 4.2)

For a degree of freedom of 16 and a 95% confidence interval, t limit is 2.120 [Fisher and Yates, 1963].

Because the value for t is lower than t limit, the two samples are identical. There is no segregation, thus no influence of MBCD substance on FR for β_2-adrenergic receptor.

4.8. **Maximal FRET ratio**

Maximal FRET ratio is dependent on the type of fluorophores. The following value is the ideal case depending of molar extinction:

maximal FR (DA 0.5/0.5) is Equ. 4.8.

$$FR_{max} = \left(\frac{\varepsilon_A}{\varepsilon_D}\right)^{-1} + 1$$

[Equ. 4.8]

For the Cy3-Cy5 pair, the ratio $\frac{\varepsilon_A}{\varepsilon_D}$ at 543 nm is 0.11 [Meyer, 2005]

Thus, maximal FR for a labelling with DA 0.5/0.5 is 10.1.

In our experiment, for a labelling with Cy3/Cy5 0.5/0.5, we obtain the following result:

FR= 1.99 ± 0.08 (0.20, 95%, 24)

The value obtained indicates that the experimental FR is under the maximal FR and thus there is no aberration for FR value.

5. Discussion

5.1. FRET techniques parameters

Calculation of cross-talks S1 and S4 was the first problem. The FR strongly depends on these parameters. A bad correction of these terms and the FR would be completely false.
The relative error obtained for FR is of 5.2%. This error is an analytic error. But, like mentioned in point 4.1, the main error is due to the intensity of fluorophores on the plasma membrane. FR varies depends on the position in plasma membrane. To obtain a Gaussian curve, only the center of plasma membrane has been kept with an intensity more or less constant.

A comparison with cross-talks of Bruno Meyer [Meyer, 2005] can be made and the result is that values are more or less similar for the same fluorophores and parameters. Based on these observations, cross-talks determined using Equ. 4.3 can be accepted. When intensities in membrane plasma for FRET channel is similar, the FR histogram has a normal distribution (Figure 4.11).

5.2. Oligomerization

This work is the first application of FRET technique to determine oligomerization degree of β_2-adrenergic receptor. However, many experiments have already applied FRET technique to other 7TM receptors, for example Neuropeptide Y_1 [Dinger et al., 2003], neurokinin-1 receptor [Meyer, 2005] or α_{1b}-adrenergic receptors. [Stanasila et al., 2003]

The measured degree of oligomerization of β_2-adrenergic receptors does not correspond to BRET experiments which have demonstrated that β_2-adrenergic receptor is a homo-dimer [Mercier et al., 2002; Angers et al., 2000].
Based on results shown in figure 4.12, degree of oligomerization is not the value n = 2. This hypothesis shows the impossibility to have β_2-adrenergic receptor like a homo-dimer.

Our results obtained with application of Equ. 4.5 for Figures 4.13 to 4.15 indicate that β_2-adrenergic receptor is an oligomer, but with a tetrameric structure and not a dimeric structure.

Another group dismisses the hypothesis that β_2-adrenergic receptor can form homodimers. They has been determined that β_2-adrenergic receptor is a monomer which form oligomer structure. [James et al., 2006]

Finally, it has been suggested that for rhodopsin receptors, lipid-mediated protein-protein interactions provide an additional mechanism that governs the association process and does tend to oligomerize, and that the membrane environment affects the extent of oligomerization. An equivalent model can be suggested for β_2-adrenergic receptor. [Periole et al., 2007]

The estimated diameter for a GPCR is 40-50 Å. [Palczewski et al., 2000; Mercier et al., 2002; James et al., 2006]
The calculated distance is 64.8 ± 1.8 Å. Thus, this distance is larger than obtained from estimations in the literature. The upper distance is another argument for the hypothesis that the β_2-adrenergic receptor is not a dimer with strong interactions, but protein-protein interactions between 4 units.

Our results bring a new lighting on β_2-adrenergic receptor. Using FRET, we demonstrate that this receptor is under form of oligomer in plasma membrane. It does not a dimer but more specifically an oligomer with 4 units.

5.3. Dependence of FRET ratio on receptor density

For GPCRs, different experiments were done on many receptors to observe the FR variation with the density of receptors and results are different between different receptors.
Meyer have demonstrated that for NK1R (neurokinin-1 receptor) MBCD has decreasing effect on FR, suggesting a model where NK1Rs are localized in lipid microdomains. [Meyer, 2005]
For others GPCRs, results are different. Between adenosine A_{2A} receptors [Canals et al., 2004] or A_{2A} and D_2Rs [Canals et al., 2003], BRET signals are no affected by the variation of receptors density.

This work shows for β_2-adrenergic receptor that for a variation of receptors of a factor 4, there is no dependence of FRET ratio on the density of receptors. Two different DA ratios give the same result (Figure 4.16 and Figure 4.17).
The degree of Oligomerization is the same for a large domain (50-300 AcRe, Figure 4.13), a reduced domain (150-200 AcRe, Figure 4.14) and the stable HEK 293 (130-450 AcRe, Figure 4.15). FR is not dependent to receptors for the studied domain.
These results show that degree of oligomerization of the β_2-adrenergic receptor does not change with the number of receptors on membrane plasma. In the analyzed domain, the β_2-adrenergic receptor forms always an oligomer with a tetrameric structure.
Domain with more receptors can not be analyzed because with a strongly concentration of receptors, cells comes spherical and it is impossible to measure FR.

Two other research groups have studied the β_2-adrenergic receptor by BRET technique.
For the first group, The BRET signal is dependent on cell expression. The BRET efficiency increases with the increase of receptors. Those authors think that BRET showed randomly interacting proteins. [James et al., 2006]. This result indicates that the β_2-adrenergic receptor is not a dimer but an oligomer.
In opposite, another group observes that between 1.4 to 26 pmol/mg β_2-adrenergic receptor expression level, BRET signal does not increase and is stable. In this domain, the β_2-adrenergic receptor concentration does not influence on the BRET signal. After this domain, we can see that the BRET level increase with the receptor level. [Mercier et al., 2002]
These results show that for a domain (1.4 to 26 pmol/mg), β_2-adrenergic receptor is a dimer. With more than 47 pmol/mg, the BRET increase from random collision between evenly dispersed donor and acceptor molecule. [Kenworthy and Edidin, 1998]

It is difficult to compare the two different groups, because in the first case there is no indication about the expression level range.
Another difficulty is the minimal level concentration (1.4 pmol/mg) is 18 fold over reported for human heart tissues (~0.080 pmol/mg) [Bristow et al., 1986]. It has not been demonstrated that β_2-adrenergic receptor is present as a dimer at physiological expression level.

In this work, our results show no dependence of FRET ratio on the density of receptors without knowing exactly what our expression level is.
We have at least a factor 4 between low and high expression without leading to any variation in the FR.

5.4. Cholesterol depletion

Contrary to NK1-receptor which shows a decreasing of FR with cholesterol depletion [Meyer, 2005], same experiment on β_2-adrenergic receptors shows no influence on the FR. This result was obtained after a T-test (Table. 4.2).
There are no microdomains in which β_2-adrenergic receptors can be concentrated with the cholesterol depletion method.
The result obtained without variation of FR for β_2-adrenergic receptors is similar than another group showed for two different GPCRs, Adenosine A_{2A} and dopamine D_2. [Canals et al., 2003; Canals et al., 2004]

Nevertheless, we should extent the result of cholesterol depletion to the influence of lipids in bilayers.
A new approach by simulations of model bilayers shows that localized adaptation of the membrane bilayer is due to the presence of receptors. These experiments were made with rhodopsin monomers. It shows that the type of lipids in the bilayer can influence FRET efficiency. This local membrane deformation appears to be a key factor defining the rate, extent and orientational preference of protein-protein association. [Periole et al., 2007]

With the result of cholesterol depletion, an assay on one day for two different samples does not show the presence of microdomains with the β_2-adrenergic receptor. More assays will must be effected to confirm this result.

6. Conclusion and Outlook

The β_2-adrenergic receptor was studied by FRET imaging in HEK 293 life cells. Results obtained shows that β_2-adrenergic receptors are present in the plasma membrane in the form of oligomers, with an interaction protein-protein of degree four (tetrameric structure). There is no dependence between expression level of receptors and FRET ratio for expression levels analyzed. Finally, cholesterol depletion indicates that there is no influence on the FR.

The next step for the β_2-adrenergic receptor, to complete results for this work, would be the estimation of receptor surface density on cells with stable HEK 293 by lipid vesicles preparation to compare our results with literature.

FRET experiment will must be done with a physiological expression level of β_2-adrenergic receptor in life cells and a temperature of 37°C for same condition as in human body. Only these conditions will can give the exactly structure of β_2-adrenergic receptors for human tissues with exactitude.

7. Bibliography

Adair, B.D. and Engelman, D.M.: Glycophorin A helical transmembrane domains dimerize in phospholipid bilayers: a resonance energy transfer study. *Biochemistry*, 33(18):5539-5544, 1994.

Alberts, B., Jonhson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P.: *Molecular biology of the cell*. Hardcover, New York, fourth edition, 2002.

Angers, S., Salahpour, A., Joly, E., Hilairet, S., Chelsky, D., Dennis, M. and Bouvier, M.: Detection of β_2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc. Natl. Acad. Sci. USA*, 97(7):3684-3689, 2000.

Breit, A., Lagacé, M. and Bouvier, M.: Hetero-oligomerization between β_2- and β_3-adrenergic receptors generates a β-adrenergic signaling unit with distinct functional properties. *J. Biol. Chem.*, 279(27):28756-28765, 2004.

Bristow, M.R., Ginsburg, R., Umans, V., Fowler, M., Minobe, W., Rasmussen, R., Zera, P., Menlove, R., Shah, P., Jamieson, S. and Stinson, E.B.: β_1- and β_2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective β_1-receptor down-regulation in heart failure. *Circ Res.,* 59: 297-309, 1986

Canals, M., Marcellino, D., Fanelli, F., Ciruela, F., de Benedetti, P., Goldberg, S.R., Neve, K., Fuxe, K., Agnati, L.F. and Woods, A.S.: Adenosine A_{2A}–dopamine D_2 receptor–receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J. Biol. Chem.,* 278:46741–46749, 2003.

Canals, M., Burgueno, J., Marcellino, D., Cabello, N., Canela, E.I., Mallol, J., Agnati, L., Ferré, S., Bouvier, M. and Fuxe, K.: Homodimerization of adenosine A_{2A} receptors: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J. Neurochem.*, 88:726–734, 2004.

Dalrymple, M.B., Pfleger, K.D.G. and Eidne, K.A.: G protein-coupled receptor dimers: Functional consequences, disease states and drug targets. *Pharmacol. Ther.*,118:359-371, 2008.

Dinger, M.C., Bader, J.E., Kobor, A.D., Kretzschmar, A.K. and Beck-Sickinger, A.G.: Homodimerization of neuropeptide Y receptors investigated by fluorescence resonance energy transfer in living cells. *J. Biol. Chem.*,278(12):10562-10571, 2003.

Erickson, M.G., Alseikhan, B.A., Peterson, B.Z. and Yue, D.T.: Preassociation of calmodulin with voltage-gated Ca^{2+} channels revealed by FRET in single living cells. *Neuron*, 31:973-985, 2001.

Fisher, R.A. and Yates, F.: *Statistical tables for biological, agricultural and medical research*. Oliver and Boyd, London, sixth edition, 1963.

Förster, T.: Zwischenmolekulare Energiewanderung und Fluoreszenz. *Annalen der Physik,* 2(1-2):55-75, 1948.

Hansen, J.L. and Sheikh, S.P.: Functional consequences of 7TM receptor dimerization. *Eur. J. Pharm. Sci.*, 23:301-317, 2004.

Held, P.: An introduction to fluorescence resonance energy transfer (FRET) technology and its application in bioscience. *BioTek*:1-8, 2005.

James, J.R., Oliveira, M.I., Carmo, A.M., Iaboni, A. and Davis, S.J.: A rigorous experimental framework for detecting protein oligomerization using bioluminescence resonance energy transfer. *Nat. Methods*, 3(12):1001-1006, 2006.

Jordan, M., Schallhorn, A. and Wurm, F.M.: Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucl. Acids Res.*, 24(4):596–601, 1996.

Kenworthy, A.K. and Edidin, M.: Distribution of a glycosylphosphatidylinositolanchored protein at the apical surface of MDCK cells examined at a resolution of < 100 Å using imaging fluorescence resonance energy transfer. *J. Cell. Biol.*, 142(1):69–84, 1998.

Lakowicz, J.R.: *Principles of fluorescence spectroscopy*. Springer, New York, third edition, 2006.

Mercier, J.F., Salahpour, A., Angers, S., Breit, A. and Bouvier, M.: Quantitative assessment of β_1- and β_2-adrenergic receptor homo- and hetero-dimerization by bioluminescence resonance energy transfer. *J. Biol. Chem.*, 277(47):44925–31, 2002.

Meyer, B.H.: *Investigation of the neurokinin-1 receptor by fluorescence techniques*. Thesis N° 3272, Ecole Polytechnique Fédérale de Lausanne, Lausanne, 2005.

Miller, J.N.: Fluorescence energy transfer methods in bioanalysis. *Analyst*, 130:265-270, 2005.

Milligan, G.: Application of bioluminescence- and fluorescence resonance energy transfer to drug discovery at G protein-coupled receptors. *Eur. J. Pharm. Sci.*, 21:397-405, 2004.

Oldham, W.M. and Hamm, H.E.: Heterotrimeric G protein activation by G protein-coupled receptors. *Nat. Rev.*, molecular *cell biology*, 9:60-71, 2008.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Le Trong, I., Teller, D.C., Okada, T., Stenkamp, R.E., Yamamoto, M. and Miyano. M.: Crystal structure of rhodopsin: A G protein-coupled receptor. *Science*, 289(5480):739–45, 2000.

Periole, X., Huber, T., Marrink, S-J. and Sakmar, T.P.: G protein-coupled receptors self assemble in dynamics simulations of model bilayers. *J. Am. Chem. Soc.*, 129:10126-10132, 2007.

Pawley, J.B.: *Handbook of biological confocal microscopy*. Springer, New York, third edition, 2006.

Pierce, K.L., Premont, R.T. and Lefkowitz, J.: Seven-transmembrane receptors. *Nat. Rev.*,3:639-650, 2002.

[*Qiagen*] Fast-forward protocol for transfection of 293 cells in 96-well plates. *Qiagen Handbooks*, TFP28:1-2, 2006.

Rasmussen, S.G.F., Choi, H-J., Rosenbaum, D.M., Kobilka, T.S., Thian, F.S., Edwards, P.C., Burghammer, M., Ratnala, V.R.P., Sanishvili, R., Fischetti, R.F., Schertler, G.F.X., Weis, W.I. and Kobilka, B.K.: Crystal structure of the human β_2-adrenergic G-protein-coupled receptor. *Nature*, 450(15):383-387, 2007.

Rice, K.G.: Application of fluorescence resonance energy transfer to analyze carbohydrates. *Anal. Biochem.*, 297:117-122, 2001.

Salahpour, A., Angers, S., Mercier, J-F., Lagacé, M., Marullo, S. and Bouvier, M.: Homodimerization of the β_2-adrenergic receptors as a prerequisite for cell surface targeting. *J. Biol. Chem.*,279(32):33390-33397, 2004.

Sekar, R.B. and Periasamy, A.: Fluorescence resonance energy (FRET) microscopy imaging of live cell protein localizations. *J. Cell. Biol.*, 160(5):629-633, 2003.

Skoog, D.A., Holler, F.J. and Crouch, S.R.: *Principles of instrumental analysis*. Thompson, Belmont, sixth edition, 2007.

Stanasila, L., Perez, J.B., Vogel, H. and Cotecchia, S.: Oligomerization of the α_{1a} and α_{1b}-adrenergic receptor subtypes: potential implications in receptor internalization. *J. Biol. Chem.*, 278(41):40239-40251, 2003.

Veatch, W. and Stryer, L.: The dimeric nature of the gramicidin A transmembrane channel: conductance and fluorescence energy transfer studies of hybrid channels. *J. Mol. Biol.*, 113(1):89-102, 1977.

Truong, K., Sawano, A., Mizuno, H., Hama, H., Tong, K., Mal, T.K., Miyawaki, A. and Ikura, M.: FRET-based in vivo Ca^{2+} imaging by a new calmodulin-GFP fusion molecule. *Nat. Struct. Biol.*, 8:1069-1073, 2001.

Wieb Van Der Meer, B., Coker, G.III and Chen, S-Y.S*.: Resonance energy transfer, theory and data*. VCH, New York, first edition, 1994.

8. Acknowledgements

First of all, I thank for Prof. Jean-Manuel Segura and Prof. Horst Vogel for giving me the opportunity to work in EPFL and particularly in department of Institute of Chemical Sciences and Engineering.

Special thank to Joachim Piguet for his support intellectual and technique and his help for different difficulties had during this diploma thesis.

I would like also like to thank Menno Tol for proofreading of his manuscript.

And finally, I thank all members of the LCPPM group for the nice atmosphere which made working.

9. Annexe

[1] ACP-β_2AR vector information

ACP-ß2AR

Responsible : Nadia Cherouati
Date : 26.09.06

Stock location

DNA stock

Location : -80 freezer, rack 1
Box name : DNA stock
Position : A7 (3.1µg/µl)

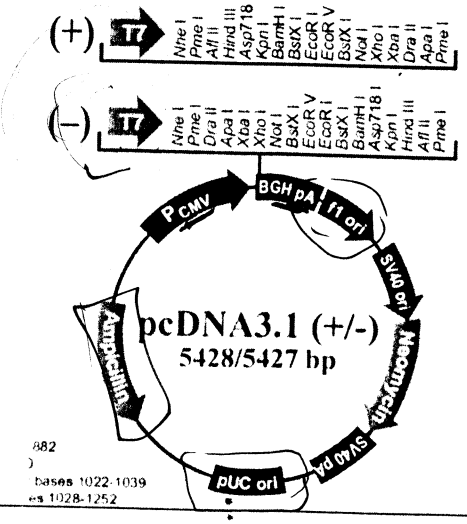
Glycerol Stock

Location : -80 freezer, rack 1
Box name : DNA Glycerol stock
Position :

Vector Information

Name : **pcDNA3.1+**
Supplier :
Size : 5428 bps
Organism : E. coli strains
Selection marker : ampicillin resistance
More info : For more vector info look in the file (file name)

Plasmid map :



Construct information

Insert

Description : signal seq5HT3-ACP-B2AR
Position : between NheI and BamHI in MCS

Expressed region

Description : **ACP-B2AR**
 # Nucleotides : 1497 bps
 # Amino acids : 497 aa
Molecular weight : 55911g/mol)

Nucleotide sequence of expressed region:

ATGCGGCTCTGCATCCCGCAGGTGCTGTTGGCCTTGTTCCTTTCCATGCTG
ACAGGGCCGGGAGAAGGCAGCCGGAGGAGGGCCACCCAGATCGATTACG
TAATGAGCACTATCGAAGAACGCGTTAAGAAAATTATCGGCGAACAGCTG
GGCGTTAAGCAGGAAGAAGTTACCAACAATGCTTCTTTCGTTGAAGACCT
GGGCGCGGATTCTCTTGACACCGTTGAGCTGGTAATGGCTCTGGAAGAAG
AGTTTGATACTGAGATTCCGGACGAAGAAGCTGAGAAAATCACCACCGTT
CAGGCTGCCATTGATTACATCAACGGCCACCAGGCGAAGCTTATGGGGCA
ACCCGGGAACGGCAGCGCCTTCTTGCTGGCACCCAATAGAAGCCATGCGC
CGGACCACGACGTCACGCAGCAAAGGGACGAGGTGTGGGTGGTGGGCAT
GGGCATCGTCATGTCTCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCT
GGTCATCACAGCCATTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACT
ACTTCATCACTTCACTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGG
TGCCCTTTGGGGCCGCCCATATTCTTATGAAAATGTGGACTTTTGGCAACT
TCTGGTGCGAGTTTTGGACTTCCATTGATGTGCTGTGCGTCACGGCCAGCA
TTGAGACCCTGTGCGTGATCGCAGTGGATCGCTACTTTGCCATTACTTCAC
CTTTCAAGTACCAGAGCCTGCTGACCAAGAATAAGGCCCGGGTGATCATT
CTGATGGTGTGGATTGTGTCAGGCCTTACCTCCTTCTTGCCCATTCAGATG
CACTGGTACCGGGCCACCCACCAGGAAGCCATCAACTGCTATGCCAATGA
GACCTGCTGTGACTTCTTCACGAACCAAGCCTATGCCATTGCCTCTTCCAT
CGTGTCCTTCTACGTTCCCCTGGTGATCATGGTCTTCGTCTACTCCAGGGT
CTTTCAGGAGGCCAAAAGGCAGCTCCAGAAGATTGACAAATCTGAGGGCC
GCTTCCATGTCCAGAACCTTAGCCAGGTGGAGCAGGATGGGCGGACGGGG
CATGGACTCCGCAGATCTTCCAAGTTCTGCTTGAAGGAGCACAAAGCCCT
CAAGACGTTAGGCATCATCATGGGCACTTTCACCCTGTGCTGGCTGCCCTT
CTTCATCGTTAACATTGTGCATGTGATCCAGGATAACCTCATCCGTAAGGA
AGTTTACATCCTCCTAAATTGGATAGGCTATGTCAATTCTGGTTTCAATCC
CCTTATCTACTGCCGGAGCCCAGATTTCAGGATTGCCTTCCAGGAGCTTCT
GTGCCTGCGCAGGTCTTCTTTGAAGGCCTATGGGAATGGCTACTCCAGCA
ACGGCAACACAGGGGAGCAGAGTGGATATCACGTGGAACAGGAGAAAGA
AAATAAACTGCTGTGTGAAGACCTCCCAGGCACGGAAGACTTTGTGGGCC
ATCAAGGTACTGTGCCTAGCGATAACATTGATTCACAAGGGAGGAATTGT
AGTACAAATGACTCACTGCTGTAA

Amino acid sequence of expressed region:

IDYVMSTIEERVKKIIGEQLGVKQEEVTNNASFVEDLGADSLDTVELVMALEE
EFDTEIPDEEAEKITTVQAAIDYINGHQAKLMGQPGNGSAFLLAPNRSHAPDH
DVTQQRDEVWVVGMGIVMSLIVLAIVFGNVLVITAIAKFERLQTVTNYFITSL
ACADLVMGLAVVPFGAAHILMKMWTFGNFWCEFWTSIDVLCVTASIETLCVI
AVDRYFAITSPFKYQSLLTKNKARVIILMVWIVSGLTSFLPIQMHWYRATHQE
AINCYANETCCDFFTNQAYAIASSIVSFYVPLVIMVFVYSRVFQEAKRQLQKI
DKSEGRFHVQNLSQVEQDGRTGHGLRRSSKFCLKEHKALKTLGIIMGTFTLC
WLPFFIVNIVHVIQDNLIRKEVYILLNWIGYVNSGFNPLIYCRSPDFRIAFQELL
CLRRSSLKAYGNGYSSNGNTGEQSGYHVEQEKENKLLCEDLPGTEDFVGHQ
GTVPSDNIDSQGRNCSTNDSLL*

Sequencing results

Antisense primer : pCR3.1-BGHrev
Primer sequence : TAG AAG GCA CAG TCG AGG
Antisense sequence :

CTCTAGACTCGAGCGGCCGCCACTGTGCTGGATATCTTATTAGTGATGGTG
ATGGTGATGGTGATGGTGATGGAATTCCTTATCGTCATCGTCCTTGTAGTC
GGATCCCAGCAGTGAGTCATTTGTACTACAATTCCTCCCTTGTGAATCAAT
GTTATCGCTAGGCACAGTACCTTGATGGCCCACAAAGTCTTCCGTGCCTGG
GAGGTCTTCACACAGCAGTTTATTTTCTTTCTCCTGTTCCACGTGATATCCA
CTCTGCTCCCCTGTGTTGCCGTTGCTGGAGTAGCCATTCCCATAGGCCTTC
AAAGAAGACCTGCGCAGGCACAGAAGCTCCTGGAAGGCAATCCTGAAAT
CTGGGCTCCGGCAGTAGATAAGGGGATTGAAACCAGAATTGACATAGCCT
ATCCAATTTAGGAGGATGTAAACTTCCTTACGGATGAGGTTATCCTGGATC
ACATGCACAATGTTAACGATGAAGAAGGGCAGCCAGCAGAGGGTGAAAG
TGCCCATGATGATGCCTAACGTCTTGAGGGCTTTGTGCTCCTTCAAGCAGA
ACTTGGAAGATCTGCGGAGTCCATGCCCCGTCCGCCCATCCTGCTCCACCT
GGCTAAGGTTCTGGACATGGAAGCGGCCCTCAGATTTGTCAATCTTCTGG
AGCTGCCTTTTGGCCTCCTGAAAGACCCTGGAGTAGACGAAGACCATGAT
CACCAGGGGAACGTAGAAGGACACGATGGAAGAGGCAATGGCATAGGCT
TGGTTCGTGAAGAAGTCACAGCAGGTCTCATTGGCATAGCAGTTGATGGC
TTCCTGGTGGGTGGCCCGGTACCAGTGCATCTGAATGGGCAAGAAGGAGG
TAGGCCTGAC

Sense primer : T7
Primer sequence : (TAA TAC GAC TCA CTA TAG GG)
Sense sequence :

CCCATGCGGCTCTGCATCCCGCAGGTGCTGTTGGCCTTGTTCCTTTCCATG
CTGACAGGGCCGGGAGAAGGCAGCCGGAGGAGGGCCACCCAGATCGATT
ACGTAATGAGCACTATCGAAGAACGCGTTAAGAAAATTATCGGCGAACA
GCTGGGCGTTAAGCAGGAAGAAGTTACCAACAATGCTTCTTTCGTTGAAG
ACCTGGGCGCGGATTCTCTTGACACCGTTGAGCTGGTAATGGCTCTGGAA
GAAGAGTTTGATACTGAGATTCCGGACGAAGAAGCTGAGAAAATCACCA
CCGTTCAGGCTGCCATTGATTACATCAACGGCCACCAGGCGAAGCTTATG
GGGCAACCCGGGAACGGCAGCGCCTTCTTGCTGGCACCCAATAGAAGCCA
TGCGCCGGACCACGACGTCACGCAGCAAAGGGACGAGGTGTGGGTGGTG
GGCATGGGCATCGTCATGTCTCTCATCGTCCTGGCCATCGTGTTTGGCAAT

GTGCTGGTCATCACAGCCATTGCCAAGTTCGAGCGTCTGCAGACGGTCAC
CAACTACTTCATCACTTCACTGGCCTGTGCTGATCTGGTCATGGGCCTGGC
AGTGGTGCCCTTTGGGGCCGCCCATATTCTTATGAAAATGTGGACTTTTGG
CAACTTCTGGTGCGAGTTTTGGACTTCCATTGATGTGCTGTGCGTCACGGC
CAGCATTGAGACCCTGTGCGTGATCGCAGTGGATCGCTACTTTGCCATTAC
TTCACCTTTCAAGTACCAGAGCCTGCTGACCAAGAATAAGGCCCGGGTGA
TCATTCTGATGGTGTGGATTGTGTCAGGCCTTACCTCCTTCTTGCCCATTCA
GATGCACTGGTACCGGGCCACCCACCAGGAAGCCATCAACTGCTATGCCA
ATGAGACCTGCTGTGACTTCTTCACGAACCAAGCCTATGCCATTGCCTCTT
CCATCGTGTCCTTCTACGTTCCCTGGTGATCATGGTCTTCGTCTACTCCAG
GGTCTTTCAGAGGCAAAAGGCAGCTCAGAAGATT