

# FRET as a Tool to Study G-protein Coupled Receptor Oligomerization in HEK Cells.

**Tobias Haase<sup>1</sup>, Stefanie Kamann<sup>1</sup> and Angela Brüx<sup>c\*</sup>**

<sup>1</sup>Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Charité-Universitätsmedizin Berlin, Campus Virchow Klinikum, Augustenburger Platz 1, 13353 Berlin

<sup>c</sup>Communicated by BERTHOLD TECHNOLOGIES GmbH & Co. KG, Calmbacher Str. 22, D-75323 Bad Wildbad, Germany

\*Address all correspondence to: angela.bruex@berthold.com

- FRET measurement in HEK cell using the Mithras LB940 multimode microplate reader
- Oligomerization of GPCR

## Abstract

The formation of GPCR oligomers reveal a further level of molecular cross-talk between signalling molecules and has potential effects on receptor function and downstream signalling under physiological and pathological conditions. Using biophysical methods based on resonance energy transfer, it is possible to investigate the nature and roles of receptor coupling in living cells. Elucidating the impact of GPCR dimerization in the activation processes will help to investigate previously unexpected pharmacological diversities and will lead to the development of novel pharmaceutical agents that promote or inhibit GPCR dimerization. Here we report the use of fluorescence resonance energy transfer (FRET) technology in HEK cells as a model to study GPCR oligomerization.

## Introduction

An increasing number of observations showed that G-protein coupled receptors (GPCRs) exist as homo/heterodimeric and presumably higher order oligomeric structures. Several studies showed that the formation of dimeric/oligomeric GPCR complexes has an impact on their activity and can profoundly affect their signalling and pharmacology<sup>1</sup>. Although a number of biochemical and biophysical techniques had been used to study GPCR dimeri-

zation, resonance energy transfer techniques have emerged as methods of choice to study receptor coupling in living cells. Fluorescence resonance energy transfer (FRET) is based on the transfer of energy from a fluorescent donor molecule to a fluorescent acceptor molecule that leads to enhancement of acceptor emission and quenching of donor emission<sup>2</sup>. Since the efficiency of this energy transfer is dependent on the distance between donor and acceptor molecule this technique is widely used to study macromolecular interactions in living cells. Mainly these approaches are based on the fusion of fluorescence resonance energy transfer FRET-compatible GFP variants to the protein of interest. The most frequently used combination of GFP variants as FRET pair is CFP/YFP. Among chemokine receptors, which regulate a range of physiological processes, including immune cell trafficking, cancer cell migration and neuronal patterning the dimerization/oligomerization of CXC chemokine receptor 4 (CXCR4) had been extensively studied using various approaches<sup>3-6</sup>.

Here, we provide a protocol for the study of receptor dimerization using the FRET method with recombinant expressed fluorescent-tagged CXCR4 subunits. This assay allows for the determination of FRET between CFP and YFP in dependence of total expression level and donor/acceptor ratio.

## Experimental Procedures

CXCR4 cDNA was amplified from Jurkat cDNA and cloned into pECFP-N1 or pEYFP-N1 expression vectors (clontech) resulting in carboxy-terminally fluorescent-tagged CXCR4 with a six amino acid linker sequence between the receptor and the fluorescent protein. Fortyeight hours before use, HEK cells were transfected by electroporation with plas-

mids (0.01 - 4 $\mu$ g) encoding fluorescent proteins or FRET constructs. 1x10<sup>6</sup> cells were electroporated and seeded in a 6-well plate. Cells were washed with prewarmed PBS then detached from the plate and resuspended in 200  $\mu$ L PBS. One hundred  $\mu$ l of the cell suspension were transferred to a black 96-well plate and the plate was placed in the Mithras LB 940 instrument. The emission output from each well was measured sequentially in the donor-, acceptor- and FRET channel. Measurements were done in duplicate.

## Material

- Mithras LB 940 multimode microplate reader, BERTHOLD TECHNOLOGIES
- Excitation Filters: 430/10 nm (39500), 485/14 nm (40271)
- Emission Filters: 480/20 nm (39450), 530/25 nm (39451)
- 96 well plates, black
- Cells: transiently transfected HEK 293 cells
- DNA: pECFP-N1, pEYFP-N1 (Invitrogen), fluorescent-tagged constructs were made using standard molecular biology techniques
- Standard medium, buffers, etc. was used from various manufactures.

## Instrument Settings & Data Analysis

Fluorescence emission was detected in the fluorescence scanning mode, where 10x10 points of each well were scanned and averaged (figure 1). The scanning mode was chosen due to the clustering of the cells (figure 2). ECFP fluorescence was measured after excitation at 430 nm through 480/20 nm emission filter (donor channel; D). EYFP fluorescence was measured after excitation at 485 nm through 530/20 nm emission filter (acceptor channel; A). Accordingly FRET signal was detected after excitation at 430 nm through the 530/20 nm emission filter (FRET channel; F). These chosen filter settings showed the lowest signal/noise ratio in preliminary tests. To determine bleed through factors all three channels were measured using cells only expressing donor or acceptor proteins. The obtained fluorescence emission was background sub-

tracted in each channel using non-transfected cells.

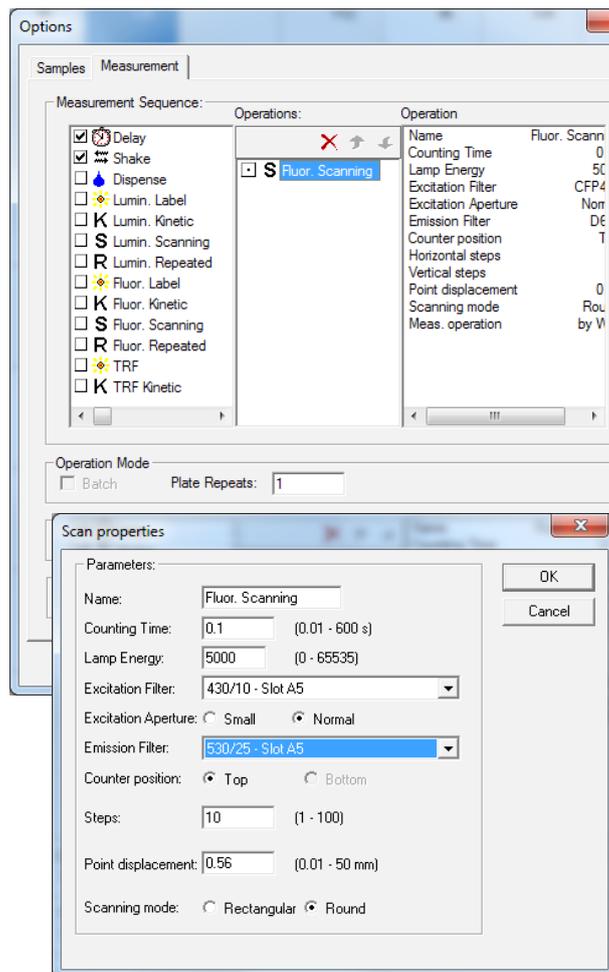


Figure 1: Screenshot of Measurement Settings

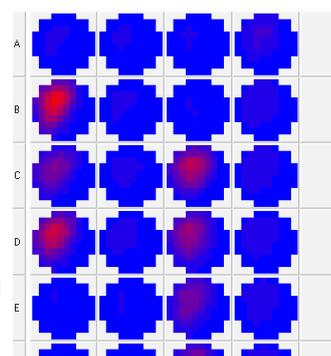


Figure 2: Screenshot of MikroWin - showing measurement result in a graphical way. It is clearly visible that signal clusters due to clustering of cell culture.

Data were collected using the MikroWin2000 software. FRET efficiency was calculated as described in Youvan et al.<sup>7</sup>, Periasamy et al.<sup>8</sup>. Briefly, bleed through factors were determined by measuring cells expressing only donor- (ECFP) or acceptor- (EYFP) fluorescent protein in all three channels and quantifying the fluorescence intensity ratio  $F_D/D_D$  and  $F_A/A_A$  for the donor- (d) and acceptor-bleed through (a), respectively:

$d = F_D / D_D$  (with  $F_D$  = FRET channel emission, only donor expressing cells;  $D_D$  = Donor channel emission, only donor expressing cells)

$a = F_A / A_A$  (with  $F_A$  = FRET channel emission, only acceptor expressing cells;  $A_A$  = Acceptor channel emission, only acceptor expressing cells).

Obtained bleed through factors were approximately 35% for ECFP ( $d$ ) and 3% for EYFP ( $a$ ). From the emission detected in the three channels and the calculated spillover factors the corrected FRET ( $F_C$ ) was calculated as follows:

$$F_C = F_{DA} - (D_{DA} \cdot d) - (A_{DA} \cdot a)$$

Since the corrected FRET depends on the total expression level of fluorescent proteins normalization of FRET was done by using calculations from Periasamy et al.<sup>8</sup> (E%; FRET efficiency) as follows:

$$E\% = 1 - [D_{DA} - (D_{DA} + F_C \cdot Q_d / Q_a)]$$

where  $F_C$  is the corrected FRET from the equation above and  $D_{DA}$  corresponds to the background subtracted fluorescence intensities acquired through the donor channel,  $Q_d$  and  $Q_a$  are the donor and acceptor quantum yields, respectively ( $Q_d=0.4$ ,  $Q_a=0.61$ ).

For FRET titration experiments, FRET efficiencies were expressed as a function of the YFP/CFP concentration (type I experiment) or total YFP fluorescence (type II experiment)<sup>9</sup>. YFP/CFP ratio was calculated using the background subtracted YFP emission ( $A_{DA}$ -Background) and  $F_C + D_{DA}$  (corrected FRET emission + emission donor channel) reflecting the unquenched CFP emission<sup>8</sup>. The obtained values were then adjusted to the emission ratio of the positive control, an YFP-CFP fusion protein, connecting the two fluorescent proteins with a fixed 1:1 stoichiometry via a six amino acid linker.

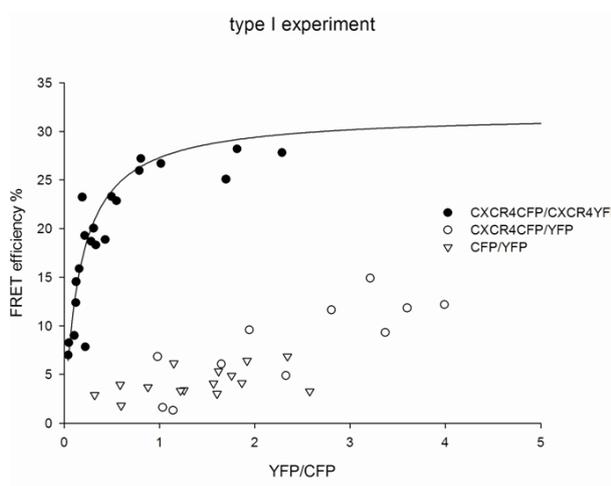
## Results

Recombinantly expressed fusion proteins often accumulate in intracellular compartments making it difficult to discriminate whether FRET results from a specific interaction of oligomers or from randomly

interacting fluorescent proteins. Moreover, the energy transfer is depending on several factors including the distance between FRET partners, donor-/acceptor expression levels and subunit affinity. In order to correctly quantify FRET with regard to the mentioned disturbing factors, we used a protocol originally designed for BRET measurements by James et al.<sup>9</sup>.

To determine FRET emission induced by random interactions of fluorescent proteins naked CFP and YFP plasmids were co-transfected. Cells expressing just these two fluorophores showed a much lower FRET efficiency than cells expressing CXCR4-fluorophore fusion proteins (Figure 2, Figure 3). Additionally an YFP-CFP fusion protein was used as a positive control for FRET (Figure 3).

For the first set of experiments (type I experiments), a constant amount of CXCR4-CFP expression vector was co-transfected with increasing amounts of CXCR4-YFP encoding plasmid. The FRET efficiency was plotted as a function of the YFP/CFP ratio (Figure 3) showing that for CXCR4 subunits rising YFP/CFP ratios increase the FRET efficiency to saturation. This is expected for directly interacting oligomers. In contrast, the FRET efficiency was significantly lower and varied linearly with the YFP/CFP ratio in cells co-expressing CFP or CFP tagged receptor with YFP, as expected for nonspecific random interactions.



**Figure 3:** Oligomerization of fluorescent-tagged CXCR4 in dependence of the acceptor/donor ratio (Type 1 experiment). Constant amounts of CFP or CFP-tagged receptor were co-transfected with increasing amounts of YFP or YFP tagged receptors.

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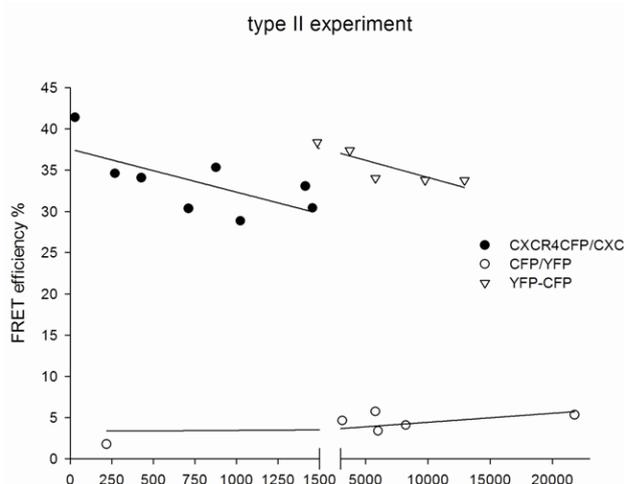
For the second set of experiments (type II experiments) the YFP/CFP ratio was kept constant while the total expression level was varied by transfecting increasing amounts of expression plasmids. The FRET efficiency was plotted as a function of YFP fluorescence since the YFP fluorescence is, in contrast to the CFP fluorescence, independent of quenching caused by energy transfer. In these type II experiments the FRET efficiency is expected to be independent of the expression level of fluorophores. Figure 4 demonstrates that the positive FRET control as well as the co-expressed CXCR4 variants show significant higher FRET efficiencies than co-expressed CFP/YFP. FRET efficiency of the negative control slightly increases with the overall expression level as expected for non-specific interactions whereas the positive control and the CXCR4 oligomers have FRET efficiencies independent of the expression level. It is noteworthy that FRET efficiencies of the positive control and CXCR4 slightly decrease with increasing expression level. These results differ from that of James et al.<sup>9</sup> where oligomers show slightly increasing FRET efficiencies with increasing expression levels. These may be due to differences between the BRET and FRET method.

## Conclusion

The described FRET assay allows the detection of intermolecular FRET in dependence of total expression levels and donor/acceptor ratio. Using this method it is possible to screen for interacting proteins and analyse subunit affinity (hillslope) as well as conformational changes that influence the distance between fluorescent proteins.

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**Figure 4:** Oligomerization of fluorescent-tagged CXCR4 is dependent of total expression level (Type 2 experiment). The YFP/CFP ratio was kept constant while the total expression level was increased, reflected by YFP fluorescence.