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▶ To cite this version:

Damien Maurel, Julie Kniazeff, Gérard Mathis, Eric Trinquet, Jean-Philippe Pin, et al.. Cell surface detection of membrane protein interaction with homogeneous time-resolved fluorescence resonance energy transfer technology. Analytical Biochemistry, 2004, 329 (2), pp.253-62. 10.1016/j.ab.2004.02.013 hal-00318979

HAL Id: hal-00318979 https://hal.science/hal-00318979v1

Submitted on 5 Sep 2008

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Cell surface detection of membrane protein interaction with HTRF® technology

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Abstract:

Direct or indirect interactions between membrane proteins at the cell surface play a central role in numerous cell processes, including possible synergistic effects between different types of receptors. Here we describe a method and tools to analyze membrane protein interaction at the surface of living cells. This technology is based on the use of specific antibodies directed against each partner and labeled either with europium cryptate or Alexa Fluor® 647. This allows the measurement of a FRET signal in a time resolved manner if both antibodies are in close proximity. This approach is here validated using the heterodimeric GABA_B receptor as a model. We show that after washing out the unbound antibodies, the time-resolved FRET (TR-FRET) signal can be measured together with the expression level of both partners via the quantification of the donor and acceptor fluorophores bound to the cells. Thanks to the high sensitivity of this method and to the low concentration of antibodies required, we show that the signal can also be measured directly after the incubation period without washing out the unbound antibody (Homogeneous Time Resolved FRET, HTRF[®]). As such this method is highly sensitive, reproducible and compatible with the development of high throughput screening protocols.

Introduction

Protein-protein interactions, either direct or indirect, play a central role in numerous cellular processes. Although this is the subject of intense research for intracellular proteins, less is known for the membrane proteins at the cell surface. Indeed, a number of regulatory processes have been recently shown to result from the direct association of membrane proteins, either via their direct interaction or through intracellular scaffolding proteins [1-6]. Such interactions likely have important consequences in the integration of simultaneous extracellular signals via synergistic effects between the activities of these interacting proteins. It is therefore of importance to develop tools and technologies that can be used to analyze selectively interactions between membrane proteins at the cell surface.

Up to now, interactions between membrane proteins have been examined through biochemical approaches such as migration of protein complexes in SDS page, or co-immunoprecipitation studies [7]. Enzyme complementation assays have also been used successfully to demonstrate protein-protein interaction in living cells [8]. Within the last few years, FRET methodologies are being commonly used to measure molecular proximity. These methods need labeling of target proteins with a pair of donor and acceptor fluorophores. This can be achieved either by genetic fusion of a cDNA encoding a fluorescent protein [9, 10], or direct chemical labeling of the proteins [11]. So far, the most commonly used methods take advantage of either the cyan and yellow variants of the green fluorescent protein (GFP) for FRET (FRET-GFP) [12], or luciferase and GFP for Bioluminescence Resonance Energy Transfer (BRET) [13]. Although the FRET-GFP assay is well suited for imaging [14], it is not sensitive enough for direct measurement in culture wells, as requested for high throughput screening (HTS) protocols. Whereas the BRET assay can be used in such a multi-well plate format, the addition of coelenterazin to stimulate luciferase at a precise time before reading the signal impairs the use of this method in HTS. Finally, all these methodologies measured the association of the studied proteins in all cell compartments, leading to the possible observation of membrane protein-protein association resulting from their clustering during their trafficking to or from the plasma membrane, but not necessarily from their association at the cell surface.

Recently, fluorophore-conjugated antibodies specific for extracellular epitopes (either native or engineered) of membrane proteins were used to visualize protein interaction at the cell surface of intact cells. Patel's group used antibodies labeled with fluorescein and rhodamine to monitor the association of various types of GPCRs at the cell surface [15, 16]. However, FRET between these two fluorophores could only be measured using photobleaching of the acceptor molecule, a technique not suitable for rapid measurements in a multi-well plate format. A second group used europium-chelate and an allophycocyanin protein (XL665) conjugated antibodies [17]. The long emission lifetime allows a time-resolved measurement of the FRET signal (TR-FRET). This avoids any signal contamination generated by shorter-lived signals and makes this system very sensitive. However the washing steps needed to extract the specific signals from the large background resulting from the use of high concentration of antibodies, limited its usefulness in high-throughput analysis of membrane protein interaction.

In the present study, we examined whether the Homogenous Time Resolved FRET (HTRF®) technology [18] (measurement of the TR-FRET signal at the end of the incubation period without washing out the unbound antibodies) using europium cryptate and Alexa Fluor® 647, or europium cryptate and XL665-anti tag labeled antibodies can be used to visualize membrane proteins interactions at the surface of living cells. To that aim we used the γ -aminobutyrate B (GABA_B) receptor as a model system since this GPCR has been reported to form obligatory heterodimers composed of the GABA_{B1} and GABA_{B2} subunits [19]. Indeed, the GABA_{B1} subunit involved in agonist binding [20, 21] is naturally retained in the endoplasmic reticulum (ER) when expressed alone. The GABA_{B2} subunit, responsible for the G-protein activation [22,

23], unmasks the ER retention of $GABA_{B1}$ and thus allows the expression of the heterodimer at the cell surface [24, 25].

We show here the reproducibility and the specificity of the TR-FRET method that allows the quantification of the FRET signal and the expression level of both partners in the same well. We also show that the method is sensitive enough for HTRF® measurements, making it compatible with the development of HTS assays. Recently, this technology has been used to elucidate the molecular determinants involved in the allosteric control of agonist affinity within the dimeric GABA_B receptor [26].

Materials and Methods

Culture and Transfection of Human Embryonic Kidney (HEK 293) cells

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen SARL) supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin, 100 U/ml final). The plasmids expressing the rat V₂ vasopressin receptor [27], the GABA_{B1} and GABA_{B2} receptor subunits [22, 23] carrying either a Haemagglutin (HA) or a myc epitope at their N-termini were described previously. Electroporation was performed in a volume of 300 μ l with a total of 10 μ g of DNA (GABA_B-subunits 1 and 2 plasmid DNA (2 μ g each), V₂ receptor (1 μ g) and carrier DNA (pRK6 to a final amount of 10 μ g)), and 10 million cells in electroporation buffer (50 mM K₂HPO₄, 20 mM CH₃COOK, 20 mM KOH, pH 7.4). After electroporation (260 V, 1 mF, Bio-Rad Gene Pulser electroporator; Bio-Rad Laboratories, Hercules, CA), cells were resuspended in DMEM supplemented with 10% fetal calf serum and antibiotics in culture dishes for 24 hrs.

Antibodies and labeling

The monoclonal anti-myc antibody, 9E10 (ATCC no. CRL-1729) and monoclonal anti-HA antibody, 12CA5 mAb provided by Cis Bio Intl Research were labeled in house with the different fluorescent donors or acceptors. The donor was a Pyridine-BiPyridine europium cryptate (europium cryptate-PBP). Such europium cryptate allows the development of HTRF assays without the addition of fluoride ions in the reaction buffer. Such ions prevent any potential fluorescence quenching effect from media [28]. However, they have to be avoided in cellular assays to ensure a perfect cellular integrity. A maleimide derivative of the europium cryptate-PBP was used to label the different antibodies as the following.

Both anti-HA and anti-myc antibodies were activated with 8 equivalents of N-Succidimidyl 3-[2-pyridylthio]propionate (SPDP) (Pierce) previously dissolved in ethanol (FLUKA). After a 30 min incubation at room temperature, DTT (Sigma) was added at the final concentration of 10 mM in the antibody solution. Reduction step was incubated 15 min at room temperature, followed by an activated antibody purification on a Sephadex-G25 SF HR10/10 column (Pharmacia) pre-equilibrated with a 0.1 M phosphate buffer pH 7.0. An antibodies labeling with europium cryptate-PBP was done with a 12 fold excess of europium cryptate-PBP per antibody. Coupling reactions were incubated for 24 hrs at $+4^{\circ}C$. Europium cryptate-PBP conjugates were purified on a G25 SF HR10/30 column (Amersham Pharmacia Biotech) pre-equilibrated with a 0.1 M phosphate buffer pH 7.0. The number of europium cryptate-PBP conjugates were purified on a G25 SF HR10/30 column (Amersham Pharmacia Biotech) pre-equilibrated with a 0.1 M phosphate buffer pH 7.0. The number of europium cryptate-PBP conjugates were purified and 317 nm, and inserting the measured values into the following equation:

where the molar extinction coefficients of the europium cryptate-PBP ($\varepsilon_{europium cryptate-PBP}$) was taken as 18,000 M⁻¹.cm⁻¹, the molar extinction coefficients of the antibodies was taken to be 210,000 M⁻¹.cm⁻¹. The factor A expressed the ratio (OD_{317nm}/OD_{280nm}) for europium cryptate-PBP and was determined to be 2. Molar ratio was determined to be 7 for the anti-HA.

The acceptors used were Alexa Fluor® 647 (Molecular Probes) [29] or XL665. XL665 conjugates were synthesized as previously described [30]. A Nhydroxysuccinimide ester (NHS) derivative of the Alexa Fluor® 647 was used to label the different antibodies previously dissolved in a carbonate buffer 0.1 M pH=9. An excess of 6 Alexa Fluor® 647 per antibody was used in the labeling reaction during 30 min at room temperature. Final conjugates were purified using a G25 SF HR10/10 column (Amersham Pharmacia Biotech). The final number of dyes per antibodies was determined spectrophotometrically as described above for the europium cryptate PBP conjugates. OD at 317 nm was replaced by an OD at 650 nm, which is the maximum of absorption for Alexa Fluor® 647. The molar extinction coefficient of the Alexa Fluor® 647 at 650 nm was taken as 240,000 M^{-1} .cm⁻¹ and the factor A expressed the ratio (OD_{650nm}/OD_{280nm}) for Alexa Fluor® 647 and was determined to be 30. Molar ratios were determined to be 3 for the anti-HA conjugate and 3 for the anti-myc conjugate.

Time-resolved Fluorescence measurements and Data analysis

Fluorescence Emissions were monitored both at 620 nm and at 665 nm. A 400 μ sec integration time was used after a 50 μ sec delay to remove from the specific signal the short-lived fluorescence background. These were measured on a dual wavelength (665/620 nm) time-resolved fluorimeter (RubyStar, BMG Labtechnologies) equipped with a nitrogen laser as excitation source (337 nm). Fluorescence collected at 620 nm in the TR-FRET assay was defined as to be the total europium cryptate signal. For TR-FRET or HTRF assays, the Ratio R=[(fluorescence 665 nm/ fluorescence 620 nm) x 10⁴] was computed. Assay signals were expressed using two parameters: Delta 665 or Delta F.

Delta 665=(Positive energy transfer signal at 665 nm) - (negative signal at 665 nm). It represents the signal released by the acceptor after the FRET process.

The specific signal over background called Delta F was calculated using the following formula: Delta F=(Rpos - Rneg)/(Rneg). Rneg corresponded to the ratio for the negative energy transfer control whereas Rpos corresponded to the ratio for the positive energy transfer control. Two types of negative controls were used when two distinct antibodies (anti-HA and anti-myc, for example) were used. First, 1 μ M of unlabeled anti-myc monoclonal antibodies (9E10) was added. Second, HEK293 cells were incubated with the donor-labeled antibodies only. When both controls were performed in parallel, ratios of the fluorescence measured at 665 nm over that measured at 620 nm after excitation at 337 nm were similar. Only the second type of

negative control was then performed in every experiments thereafter. When FRET signals were measured with a single type of antibody (a 1:1 mixture of anti-HA antibody labeled with the donor fluorophore, and anti-HA antibody labeled with the acceptor fluorophore), the negative control was measured using a 1:1 mixture of donor-labeled and unlabeled antibodies.

Ro calculation for the FRET pair europium cryptate-PBP and the Alexa Fluor® 647 was done as described [30], and determined as to be ~65Å.

Measurement of the Alexa Fluor® 647 fluorescence

The total fluorescence emitted by the Alexa Fluor® 647 conjugates in the TR-FRET assay was measured using an Analyst[™] reader (Molecular Devices) equipped with the appropriate filters set (XF47 from Omega Optical). The Alexa Fluor® 647 fluorescence was detected at 682 nm using a 640 nm excitation.

TR-FRET experiments

Cells were harvested 24 h after transfection using dissociation solution (SIGMA). Trypan blue viability test and counting were routinely performed after resuspension of transfected cells. An incubation under circle rotator (different time performed and described in the figure 2) was performed at 4°C with 200,000 cells in a total volume of 200 μ l containing 1 nM europium cryptate-PBP-labeled antibody and 3 nM Alexa Fluor® 647-labeled antibody in PBS supplemented with SVF 25%. Cells were then washed, resuspended in 100 μ l PBS and distributed into a 96 well microtiter plate. The different fluorescence measurements were determined as described above.

HTRF[®] measurement

Twenty four hrs after transfection, 200,000 cells resuspended in 100 μ l PBS supplemented with SVF 25% and containing 1 nM europium cryptate-PBP- and 3 nM

Alexa Fluor® 647-labeled antibodies were dispatched into 96 well microtiter plates. After the indicated time at 4°C (to prevent protein clustering and to limit the antibodies aspecific fixation) the FRET signal was directly measured as described above.

Ligand-binding assay

A ligand-binding assay on intact HEK293 cells was performed as described previously using 0.1 nM of [¹²⁵I]-CGP64213 [23] with 1 mM GABA being used to determined the non specific binding.

Determination of inositol phosphate accumulation

Determination of inositol phosphate (IP) accumulation in transfected cells was performed in a 96 well plate format after labeling the cells overnight with *myo*-[³H]inositol (23.4 Ci/mol) as previously described [31].

Results

As a protein model system, we choose the GABA_B receptor because of its heteromeric nature, allowing the unambiguous labeling of each subunit (Fig. 1). The formation of this heterodimer was previously demonstrated after transient expression of these two subunits in HEK 293 cells as shown by coimmunoprecipitation experiments and functional coupling of the reconstituted receptor to G-proteins [23]. Moreover, the wild-type GABA_{B1} does not reach the cell surface in HEK 293 cells unless co-expressed with GABA_{B2} or mutated in its ER retention signal (GABA_{B1}-ASA) offering the additional possibility to check that only surface proteins are detected [24, 25]. Finally, insertion of either a myc or HA epitope after the signal peptide did not affect the expression and function of these subunits [23].

TR-FRET experiments

In a first set of experiments we examined whether the TR-FRET technology could be applied with the chosen tools to monitor the existence of GABA_B heterodimers. No specific fixation of the HA antibody was observed on cells expressing the HA-GABA_{B1} subunit alone, as expected since this subunit is known not to reach the cell surface in the absence of GABA_{B2} (Fig. 2). In agreement with this conclusion, specific labeling of the cells with the anti-HA-europium cryptate-PBP antibody was obtained in the absence of GABA_{B2} with a HA-GABA_{B1} mutant (GABA_{B1}-ASA) in which the ER retention signal (RSRR) is mutated into ASAR [24]. This confirms binding of the antibody relies on the presence of an extracellular epitope, such that only cell surface proteins can be detected (Fig. 2). Cells transiently expressing HA-GABA_{B1} and myc-GABA_{B2} were used to determine the binding conditions for the antibodies. We found that concentrations as low as 1 and 3 nM for the anti-HA-europium cryptate-PBP (donor) and anti-myc-Alexa Fluor® 647 (acceptor) antibodies, respectively, were sufficient to get a large and specific signal for each fluorophore on intact cells at 4°C. The detected fluorescence reached a plateau after 8 hrs incubation followed by cell washing, suggesting the saturation of the specific recognition sites of the conjugates (Fig. 3A, 3B). Moreover, the specific signal over background Delta F generated between the donor and the acceptor (Fig. 3C) in close proximity remains stable for at least 24 hrs at 4°C. Similar data were obtained if XL665 was used as an acceptor fluorophore instead of Alexa Fluor® 647 (data not shown).

We then compared the quantity of fluorescently labeled antibodies HA-GABA_{B1} at the cell surface, with the binding of the non-permeant GABA_{B1} radioligand [¹²⁵I]-CGP64213. The amount of HA-GABA_{B1} at the cell surface was controlled by the quantity of GABA_{B2}. For this reason we co-expressed various amount of myc-GABA_{B2} subunit with a fixed amount of HA-GABA_{B1}. As shown in Fig. 4, a perfect correlation was found between both signals indicating that the signal generated by the bound antibody is directly proportional to the amount of GABA_B receptors at the cell surface.

Under similar conditions, the FRET signal between the bound HA-europium cryptate-PBP and anti-myc-Alexa Fluor® 647, as determined either by the Delta F (Fig 5), or by the fluorescent signal at 665 nM (data not shown), was directly proportional to the amount of $GABA_{B1}$ at the cell surface measured with [¹²⁵I]-CGP64213 binding on intact cells. This confirms that the $GABA_B$ receptor is a heteromer at the cell surface, and that this heteromer formation is not dependent on the amount of subunits at the cell surface, at least within the range of receptor density examined here.

The presence of both anti-HA and anti-myc antibodies on the GABA_B receptor subunits may possibly affect the changes in conformation required for ligand binding or activation of the receptor. However this is very unlikely since the same amount of [¹²⁵I]-CGP64213 binding was measured on intact cells expressing both subunits before

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or after pre-incubation with saturating concentrations of both antibodies (each at 45 nM incubated overnight at 4°C) (Fig. 6A). Moreover, such a cell treatment did not prevent the GABA_B receptor from activating the chimeric G-protein Gaqi9 that allows it to stimulate IP formation (Fig. 6B).

In order to verify further the specificity of the FRET signal, we examined whether such a signal can be generated with antibodies recognizing two membrane proteins that do not interact. In a first experiment, HA-GABA_{B1} and non-tagged GABA_{B2} were co-expressed in a first set of cells, and non-tagged GABA_{B1} was coexpressed with myc-GABA_{B2} in a second set of cells. No FRET signal was measured when both populations of cells where co-cultured in the same wells (data not shown). Moreover, we also co-expressed the HA-GABA_{B1}-ASA mutant that can reach the cell surface alone, and the vasopressin V₂ receptor tagged at its N-terminal end with a myc epitope, since these two receptors have been reported not to heterodimerize [32]. As shown in Fig. 7, a very small FRET signal was measured when these two receptors are co-expressed in the same cells. Indeed, a 10 fold higher signal was obtained in cells expressing both HA-GABA_{B1}-ASA and myc-GABA_{B2}. This difference did not result from a differential expression level at the cell surface of these different constructs, since $myc-V_2$ and $myc-GABA_{B2}$ proteins were expressed at a similar level as revealed by measurement of the acceptor fluorescent signal bound to the cells (Fig. 7B et 7C). This small signal may well be the consequence of a possible interaction of these two receptors expressed at a high level (cell densitity estimates for both receptors is 500,000 receptors per transfected cell). Alternatively, this may be the consequence of random transfer resulting from the relatively high probability that the two receptors can be close enough due to their high cell surface density even though they do not physically interact with each other. Our previous study using chimeric GABA_B subunits in which the extracellular domains were swapped suggested that the 7TM region of GABA_{B2} could form homodimers [23, 33]. We therefore examined whether the GABA_{B1} and GABA_{B2} subunits homo-oligomerize. To

that aim, FRET signals were measured in cells expressing either HA-GABA_{B1}-ASA, or HA-GABA_{B2} using an equimolar mixture of HA-antibodies labeled either with europium cryptate-PBP or Alexa Fluor® 647. As shown in Fig. 8, a FRET signal could be detected in both cases, though smaller than that measured in cells expressing HA-GABA_{B1} and HA-GABA_{B2}. This suggests that both GABA_{B1}-ASA and GABA_{B2}, when expressed alone, can exist as homo-oligomers at the cell surface. These experiments demonstrate that membrane protein association can be detected using time-resolved FRET, after labeling each protein with a specific antibody and washing out the excess of free antibodies. However, this washing step is a strong limitation in a systematic approach for detection of membrane protein association.

HTRF Experiments

According to the high sensitivity of the TR-FRET signal generated, and to the low concentration of the antibodies required for labeling the target proteins, we examine if the same information could be obtained in a HTRF® assay, reading the signal at the end of the incubation without removing the free antibodies. To that aim, the assay signal was measured in cells transfected with HA-GABA_{B1} and various amounts of myc-GABA_{B2} plasmids, before (HTRF® mode) or after washing the free antibodies (TR-FRET mode). Fig. 9 shows that the HTRF® signal measured with Delta F within the homogeneous assay is directly proportional to the signal measured after washing. One can argue that the signal in the TR-FRET mode was found to be twice as large than in the HTRF® mode. This may be due to the removal of free residual antibodies during the washing steps in the heterogeneous assay that may increase the background. Even though the high background due to the presence of europium cryptate-PBP antibodies, the HTRF® signal was found to be directly proportional to the amount of GABA_B heterodimers at the cell surface (Fig. 10), and also to be specific since only a small HTRF® signal could be measured (10 fold lower signal) between the myc-V₂ and HA-GABA_{B1}-ASA receptors (Fig. 11). For these HTRF experiments, similar data were also obtained using XL665 as acceptor molecule instead of Alexa Fluor® 647 (data not shown).

Discussion

In the present study, we examined whether a HTRF® assay could be adapted to examine any possible interaction (direct or indirect) between plasma membrane proteins at the surface of living cells. For that purpose we used antibodies conjugated with europium cryptate-PBP or Alexa Fluor® 647 (or XL665) as donor and acceptor fluorophores, respectively. The intrinsic fluorescence properties of these fluorophores allow monitoring of a FRET signal at 665 nm in a time-resolved manner, making this system very sensitive. Moreover, the method described below requires only nanomolar quantities of antibodies allowing the measurement of the FRET signal in an homogenous way (HTRF®), making it compatible with the development of HTS assays.

In a first series of experiments, the TR-FRET signal was measured after washing out the unbound antibodies, as previously reported by others [17]. Using europium-cryptate PBP labeled HA-antibodies and Alexa Fluor® 647 labeled myc antibodies, we were able to detect oligomerization of HA-GABA_{B1} and myc-GABA_{B2} subunits in living cells in a 96 well plate format. The TR-FRET signal was observed only when the two partners were expressed in the same cells, and a 10-20 fold lower signal was measured when antibodies were directed against two transmembrane proteins known not to interact, the GABA_{B1}-ASA subunit and the vasopressin V₂ receptor [32]. This clearly demonstrates the specificity of this approach. Moreover, no binding of the HA antibodies could be detected with the wild-type HA-GABA_{B1} subunit expressed alone. Since this subunit is known not to reach the plasma membrane alone, this further demonstrates that the assay is specific for the detection of extracellular epitopes.

The TR-FRET signal measured between the $GABA_{B1}$ and $GABA_{B2}$ subunits was found to be directly proportional to the amount of $GABA_{B1}$ proteins at the cell surface. This indicates that the proportion of these subunits in a dimer is not

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dependent on their density in the plasma membrane, at least in the range of expression levels examined here. Such an observation would be consistent with the two subunits forming constitutive and stable dimers, in agreement with their association early after their synthesis, likely in the ER [24, 25]. Such an analysis also revealed a specific signal can be detected with as low as 500 cpm of [¹²⁵I]-CGP64213 bound to the cells, indicative of 10 fmol of receptors per well, demonstrating the sensitivity of this method.

Not only can heteromers be analyzed, but also homodimers. In that case we used equal amount of europium-cryptate PBP- and Alexa Fluor® 647 labeled anti-HA antibodies. This allowed us to show that $GABA_{B1}$ -ASA (the $GABA_{B1}$ mutant that can reach the cell surface alone), as well as $GABA_{B2}$ can both form homodimers at the cell surface. Since no consistent functioning of the $GABA_{B1}$ -ASA was reported [24, 25], our data show that this is not due to the inability of this subunit to exist in a homodimeric form in the plasma membrane.

Our data also revealed that bound antibodies do not affect the ligand binding and G-protein coupling of the GABA_B receptor. However, the FRET signal measured on intact cells was affected neither by agonists nor antagonists (data not shown), consistent with the notion that the GABA_B receptor is a constitutive dimer, and that ligand binding does not affect this process. Although agonist binding unlikely modifies the proportion of dimers at the cell surface, a possible large change in conformation may also result in a change in FRET signal, either due to a change in the distance between the two fluorophores, or to a change in their relative orientation. Based on the crystal structure of the dimer of ligand binding domain of the metabotropic glutamate receptor 1 (a receptor homologous to the GABA_B receptor), a large change in conformation of the dimer of extracellular domains of the GABA_B subunits is expected [34]. However, such a change in conformation is unlikely to be detected by our approach for several reasons. Firstly, several (3-7) fluorophores are usually linked to each antibody molecules, such that a positive change in the transfer efficacy between one donor and one acceptor molecule, can be compensated by a negative change for another donor/acceptor pair. Secondly, the position of the antibody on each subunit is likely highly variable during time, making more difficult the detection of conformational changes. Thirdly, the position of the fluorophore is not known, and is unlikely the same for all antibody molecules. Finally, the change in conformation may be too small compare to the Foster's radius (R_0) of the fluorophore pair to be detected by such an approach.

Taking advantage of the high sensitivity of this assay, and to the low concentration of antibodies required for saturation of the recognized epitopes, the TR-FRET signal can also be measured in an homogeneous assay - i.e. without washing out the unbound antibodies (HTRF® assay). Indeed, our data revealed that the signal measured under these conditions is even higher than that measured after washing, likely because some cells as well as some bound antibodies are lost during the washing step. Again, our data show that the signal is specific and the technique sensitive. Such a technology is therefore well suited for the rapid examination of proteinprotein interaction, and is compatible with an HTS format. Such an assay may prove to be useful for the screening of mutant subunits no longer interacting with each other, and for the screening of other transmembrane or extracellular partners of these subunits. Other biological processes such as a dynamic association of membrane proteins upon activation of specific cellular pathways or ligand occupation of any of the studied partners can also be rapidly examined with this technique. This assay may also be of interest to search for molecules able to modify the oligomerization state of transmembrane proteins. The issue of such methodology has many potential fields of applications, as illustrated by the recent elucidation of the molecular determinants involved in the allosteric control of agonist affinity within the dimeric $GABA_B$ receptor [26]. With the help of powerful and well-designed antibodies raised against extracellular epitopes of two putative partners, this HTRF® technology may be reasonably extended to wild-type native proteins. This will undoubtedly lead many researchers to investigate the mechanisms underlying numerous oligomeric assemblies.

Acknowledgements

This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS) ; the Action Concertée Incitative "Molécules et Cibles Thérapeutiques" from the french government and Cis Bio International (DIVT 2035 -CNRS 751869/00) (Marcoule, France). The authors whish also to thank K. Kaupmann and W. Froestl (Novartis Pharma, Basel, Switzerland) who allowed us to use [¹²⁵I]-CGP64213. We thank Drs. L. Prézeau and T. Durroux for constructive discussions and constant support throughout this work, and C. Vannier for her technical assistance.

Figure Legends

Figure 1

1A: Model system used for the TR-FRET and HTRF® protein-protein interaction format assays. Two subunits constitute the dimeric protein and they carry epitope tags at their N-extracellular termini. Two conjugated antibodies were used to generate a FRET signal if the tandem pair is in close proximity. Finally, the energy transfer from europium cryptate-labeled anti-tag A antibodies to Alexa Fluor® 647labeled anti-tag B antibodies was evaluated (see Materials and Methods).

1B: Incubation of the antibodies with cells in TR-FRET and HTRF® assays.

1C: FRET signal in HTRF® assays was measured without washing steps, making it compatible with the development of HTS assays.

1D: FRET signal in TR-FRET assays was measured after washing steps. The fluorescence generated by the bound antibody is directly proportional to the amount of receptors at the cell surface.

Figure 2

The binding of the HA mAb was performed on HEK 293 cells transiently transfected (mock, HA-GABA_{B1} alone, HA-GABA_{B1}-ASA or HA-GABA_{B1} and GABA_{B2}). The maximal binding (100%) corresponds to the heterodimeric GABA_B format and the nonspecific binding is revealed on the mock. No specific fixation of the HA antibody was detected on cells expressing only the HA-GABA_{B1} subunit, whereas the HA-GABA_{B1}-ASA mutant, able to reach the cell surface alone, was unambiguously detected. Histobars represent the mean +/- SD of quadruplicate of a representative experiment from three distinct experiments

Figure 3

Time course of binding of the two conjugated antibodies was measured as described in Materials and Methods in the TR-FRET format assay. We performed binding of europium cryptate-labeled HA antibodies and Alexa Fluor® 647-labeled myc antibodies on the heterodimer GABA_B receptor at the HEK 293 cell surface. The fluorescence of the donor (europium cryptate-PBP) (3A) and the fluorescence of the acceptor (Alexa Fluor® 647) (3B) were measured respectively at different times after washes. Finally, the FRET signal over background, which is assessed by the Delta F and represents the energy transfer between the two fluorophores, is determined (3C). Equilibrium is reached after 8 hrs. Each point represents the mean +/- SD of quadruplicate of a representative experiment from at least four independent transfections. The lines shown through these points were calculated nonlinear regression analysis of the data points using the Prism Software package.

Figure 4

Binding was determined as described in Materials and Methods with the nonpermeant $GABA_{B1}$ radioligand [¹²⁵I]-CGP64213. HEK 293 cells were transiently cotransfected for 24 hrs with fixed amount of HA-tagged $GABA_{B1}$ subunits and an increased of myc-tagged $GABA_{B2}$ subunits. A perfect linear correlation (R² = 0.9987) was found between binding and fluorescence signals.

Figure 5

5a, 5b: FRET signal was calculated with two different ratio methods and compared to the binding on GABA_{B1} at the cell surface. HEK 293 cells were transiently cotransfected for 24 hrs with HA-tagged GABA_{B1} subunits and various amounts of myctagged GABA_{B2} subunits. Anti-HA-europium cryptate-PBP and anti-myc-Alexa Fluor® 647 antibodies were used as performed in figure 2. Delta F was calculated as described in Materials and Methods. Linear correlation was calculated ($R^2 = 0.9733$) and reflected a direct proportion between binding on HA-GABA_{B1} with the non-permeant radioligand [¹²⁵I]-CGP64213 and fluorescence energy transfer signals between the two fluorophore on HA-GABA_{B1} and myc-GABA_{B2} in the TR-FRET format assay.

Figure 6

6A: HEK 293 cells expressing HA-GABA_{B1} and myc-GABA_{B2} were pre-incubated or not overnight at 4°C with saturating concentrations of anti-HA-europium cryptate-PBP and anti-myc-Alexa Fluor® 647 antibodies (each at 45 nM). Then the amount of [¹²⁵I]-CGP64213 binding was measured on intact cells as described in Materials and Methods. Histobars represent the mean +/- SD of quadruplicate of a representative experiment from three distinct experiments.

6B: The effect of one saturating concentration of GABA on IP formation in HEK 293 cells co-expressing the HA-GABA_{B1} and myc-GABA_{B2} and Gaqi9 is shown. Data are expressed as the IP production over the radioactivity remaining in the membrane and are means +/- SD of triplicate determinations from a typical experiment. Histobars represent IP production from pre-incubated cells as described in 6A, with or without stimulation and in presence or absence of antibodies pre-incubation.

Figure 7

7A: HEK 293 cells were transiently mock-transfected (only carrier DNA, pRK6) or co-transfected with HA-GABA_{B1}-ASA and myc-GABA_{B2}, or with HA-GABA_{B1}-ASA and myc-V₂. Percentage of Delta F witch represent the FRET signal over background in the TR-FRET format assay was measured in these respective transfected cells revealing specificity of the fluorescence resonance energy transfer on the $GABA_B$ receptor.

7B: Estimation of the receptor level carrying the HA epitope at cell surface in the co-transfected cells was performed by fluorescence measurement for the donor alone (europium cryptate-PBP coupled with HA mAb).

7C: Estimation of the receptor level carrying the myc epitope at cell surface in the co-transfected cells was performed by fluorescence measurement for the acceptor alone (Alexa Fluor® 647 coupled with myc mAb).

Figure 8

HEK 293 cells were transiently mock-transfected (pRK6) or transfected with only HA-GABA_{B1}-ASA or HA-GABA_{B2} alone. An equimolar mixture (3 nM) of HA-antibodies labeled either with europium cryptate-PBP or Alexa Fluor® 647 was applied on transfected cells. In the homodimeric format, we attempt to loose logically 50% of the fluorescent signal because 50% of the closed combined antibodies are engaged with two donors or two acceptors. As described in the figure 7, FRET signals represented by the Delta F and fluorescence estimation of the binding of each antibodies on the HA epitope were measured. Histobars represent the mean +/- SD of quadruplicate from three independent experiments.

Figure 9

HEK 293 cells were co-transfected with HA-GABA_{B1} and various amounts of myc-GABA_{B2} plasmids. After 24 hrs, the HTRF® signal was measured as described in Materials and Methods, and in the same way, after washing, the TR-FRET was performed. Correlation was performed in parallel between Delta F (R^2 = 0.9809) in these two methods.

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Figure 10

HEK 293 cells were transiently co-transfected with HA-tagged GABA_{B1} subunits and various amounts of myc-tagged GABA_{B2} subunits. Twenty four hrs later, transfected cells were incubated with anti-HA-europium cryptate-PBP and anti-myc-Alexa Fluor® 647 antibodies and the HTRF® signal was measured as described in Materials and Methods. Linear correlations were calculated between binding (10A) or fluorescence (10B) on HA-GABA_{B1} versus the percentage of Delta F in the HTRF format assay, $R^2 = 0.9807$ and $R^2 = 0.9846$ respectively.

Figure 11

HEK 293 cells were transiently mock-transfected or co-transfected with HA-GABA_{B1} and myc-GABA_{B2}, or with HA-GABA_{B1} and myc-V₂. HTRF[®] measurements were performed as described in the Materials and Methods.

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