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Quantitative Assessment of Seven Transmembrane Receptors (7TMRs) Oligomerization by Bioluminescence Resonance Energy Transfer (BRET) Technology

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1. Introduction

Seven transmembrane receptors (7TMRs; also designated as G-protein coupled receptors (GPCRs)) form the largest and evolutionarily well conserved family of cell-surface receptors, with more than 800 members identified in the human genome. 7TMRs are the targets both for a plethora of endogenous ligands (e.g. peptides, glycoproteins, lipids, amino acids, nucleotides, neurotransmitters, odorants, ions, and photons) and therapeutic drugs and transduce extracellular stimuli into intracellular responses mainly via coupling to guanine nucleotide binding proteins (G-proteins) (McGraw & Liggett, 2006).

These receptors have traditionally been viewed as monomeric entities and only more recent biochemical and biophysical studies have changed this view. The idea that 7TMRs might form dimers or higher order oligomeric complexes has been formulated more than 20 years ago and since then intensively studied. In the last decade, bioluminescence resonance energy transfer (BRET) was one of the most commonly used biophysical methods to study 7TMRs oligomerization. This technique enables monitoring physical interactions between protein partners in living cells fused to donor and acceptor moieties. It relies on non-radiative transfer of energy between donor and acceptor, their intermolecular distance (10 – 100 Å) and relative orientation. Over this period the method has progressed and several versions of BRET have been developed that use different substrates and/or energy donor/acceptor couples to improve stability and specificity of the BRET signal. This chapter outlines BRET-based approaches to study 7TMRs oligomerization (e.g. BRET saturation and competition assays), control experiments needed in the interpretation i.e. establishing specificity of BRET results and mathematical models applied to quantitatively assess the oligomerization state of studied receptors.

2. Seven transmembrane receptors (7TMRs): Structure and characteristics

Primary sequence comparisons reveal that 7TMRs share sequence and topology similarities allowing them to be classified as a super-gene family. These receptors are characterized by

seven hydrophobic stretches of 20-25 amino acids, predicted to form transmembrane α-helices. Prediction of transmembrane folding was based largely on the method proposed by Kyte and Doolitle (Kyte & Doolittle, 1982). This method plots the hydrophobicity of the amino acids along the sequence, assigning each amino acids a hydrophobicity index. By summing this index over a window of nine residues, the transmembrane sequence is postulated when index reaches the value of 1.6 for a stretch of ~20 amino acids. This number is based on the assumption that the membrane spanning sequences of protein are α-helical and that about six helical turns are required to span the lipid bilayer (Hucho & Tsetlin, 1996). The highly hydrophobic α-helices that serve as transmembrane spanning domains (TMs) are connected by three extracellular (ECL) and three intracellular (ICL) hydrophilic loops. Amino (N)-terminal fragment is extracellular and the carboxyl (C)-terminal tail is intracellular. In the recent years this common structural topology was also confirmed by three-dimensional crystal structure of some 7TMR members (reviewed by (Salon et al., 2011)). Additionally, 7TMRs may undergo a variety of posttranslational modifications such as N-linked glycosylation, formation of disulfide bonds, palmitoylation and phosphorylation. 7TMRs contain at least one consensus sequence for N-linked glycosylation (Asn-x-Ser/Thr), usually located near the N-terminus, although there are potential glycosylation sites in the intracellular loops. They also contain a number of conserved extracellular cysteine residues, some of which appear to play a role in stabilizing the receptor's tertiary structure. An additional highly conserved cysteine can be present within the C-terminal tail of many 7TMRs. When palmitoylated, it may anchor a part of cytoplasmic tail of the receptor to the plasma membrane, thus forming the fourth ICL and controlling the tertiary structure. Consensus sequences for potential phosphorylation sites (serine and threonine residues) are located in the second and third ICLs, and in particular, in the intracellular C-terminal tail. The most obvious structural differences between the receptors in subgroups are the length of their N-terminal fragment and the loops between TMs. Originally, 7TMRs were divided into six groups, A – F; families (also known as "groups" or "classes") A, B and C included all mammalian 7TMRs. Genome projects then generated numerous new 7TM sequences and more than 800 human 7TMRs were reclassified into five families, A – E (reviewed by (Gurevich & Gurevich, 2006; Salon et al., 2011)).

Family A (also known as the rhodopsin family) is by far the largest family of 7TMRs (containing ~700 members), and includes many of the receptors for biogenic amines and small peptides. It is characterized by very short N- and C-termini as well as several highly conserved amino acids. In most cases TMs serve as the ligand-binding site. This family contains some of the most extensively studied 7TMRs, the opsins and the β-adrenergic receptors. Recent structural information for a few family A 7TMR members (e.g. rhodopsin, opsin, human β_2-adrenergic receptor, turkey β_1-adrenergic receptor, human A_{2A}-adenosine receptor, CXC chemokine receptor type 4 and D_3-dopamine receptor) confirmed an obvious conservation of the topology and seven-transmembrane architecture (Salon et al., 2011). Family B (secretin-receptor family), which has considerably fewer members i.e. 15, is characterized by a long N-terminus (>400 amino acids) containing six conserved cysteine residues that contribute to three conserved disulfide bonds, which provide structural stability, and a conserved cleft for the docking of often helical C-terminal region of the peptide ligands. Natural ligands for family B 7TMRs are all moderately large peptides, such as calcitonin, parathyroid hormone and glucagon. Family C (metabotropic glutamate family) contains 15 members that are the metabotropic glutamate receptors (mGluRs), the

Ca^{2+} sensing receptor, and the receptor for the major excitatory neurotransmitter in the central nervous system, the γ-aminobutyric acid ($GABA_B$) receptor and orphan receptors. This family has a very large N-terminal domain (>600 amino acids), which bears the agonist binding site and also a long C-tail (Kenakin & Miller, 2010; McGraw & Liggett, 2006). Notably, family C members form obligatory dimers (Kniazeff et al., 2011). Two ancillary families consist of class D (adhesion family), containing 24 members, and class E (frizzled family), with 24 members.

3. 7TMRs homo- and hetero-oligomerization

In 1983, Fuxe et al. (Fuxe et al., 1983) formulated the hypothesis about the existence of homo-dimers for different types of 7TMRs and in the same year the first demonstration of 7TMRs homo-dimers and homo-tetramers of muscarinic receptors was published (Avissar et al., 1983). However, the evidence for dimerization existed even before that. Following classical radio-ligand studies on the insulin receptor (de Meyts et al., 1973), negative cooperativity, for which dimerization is a prerequisite, has also been demonstrated for β_2-adrenergic receptor (β_2-AR) (Limbird et al., 1975) and thyrotrophin-stimulating hormone (TSH) receptor (De Meyts, 1976) binding in the early 70's, before they were shown to be 7TMRs and this issue remained controversial for over two decades. 7TMRs can be either connected to identical partner(s), which results in formation of homo-dimers (or homo-oligomers), or to structurally different receptor(s), which results in formation of hetero-dimers (hetero-oligomers). 7TMR dimerization was proposed to play a potential role in i) receptor maturation and correct transport to the plasma membrane, ii) ligand-promoted regulation, iii) pharmacological diversity (e.g. positive and negative ligand binding cooperativity), iv) signal transduction (potentiating/attenuating signaling or changing G-protein selectivity), and v) receptor internalization and desensitization (Terrillon & Bouvier, 2004). The first widely accepted demonstration of 7TMR hetero-dimerization came from the $GABA_B$ (GBBR) receptors that exclusively function in a heteromeric form (White et al., 1998).

There is now considerable evidence to indicate that 7TMRs can form and function as homo-dimers and hetero-dimers (reviewed by (Filizola, 2010; Gurevich & Gurevich, 2008a; Palczewski, 2010)) and that these dimers may have therapeutic relevance (Casado et al., 2009). Hetero-dimerization in the C family of receptors has been most extensively studied and for some experts in the field of 7TMRs the only one demonstrated to form real dimers (for recent review see (Kniazeff et al., 2011)). In this family of 7TMRs receptors hetero-dimerization is important for either receptor function, proper expression on the cell surface or enhancing receptor activity. In the most numerous family A 7TMRs dimerization was extensively studied, although with few exceptions functional role of receptor self-association is in most cases unclear. Compelling evidence for the dimerization in the family A 7TMR was only recently demonstrated *in vivo* by Huhtaniemi's group, who was able to rescue the LH receptor knockout phenotype by complementation i.e. co-expressing two nonfunctional receptor mutants in the knockout mice (Rivero-Muller et al., 2010). Members of the family B 7TMRs have also only recently been shown to associate as stable homo-dimers. The structural basis of this, at least for the prototypic secretin receptor, is the lipid-exposed face of TM4. This complex has been postulated as being important for the structural stabilization of the high affinity complex with G-protein (reviewed by (reviewed by (Kenakin & Miller, 2010)).

In addition to widespread intra-family hetero-dimerization, inter-family hetero-dimerization has also been reported, at least between both of the family A members β_2-AR and opsin and the family B member gastric inhibitory polypeptide receptor (GIP) (Vrecl et al., 2006), and between the family A serotonin 5-HT_{2A} receptors and the family C mGluR2 (Gonzalez-Maeso et al., 2008). Both types of hetero-dimers were demonstrated to be functional, either by their ability to induce cAMP production upon agonist stimulation (family A/B hetero-dimer), or by their ability to modulate G-protein coupling (family A/C hetero-dimer).

3.1 Dimerization interface

Growing experimental data support the view that 7TMRs exist and function as contact dimers or higher order oligomers with TM regions at the interfaces. In contact dimers/oligomers of 7TMRs, the original TM helical-bundle topology of each individual protomer is preserved and interaction interfaces are formed by lipid-exposed surfaces. Although domain-swap models, i.e. models in which domains TM1/TM5 and TM6/TM7 would exchange between protomers, have also been proposed in the literature, there is there is limited direct evidence that supports these assumptions. On the other hand, compelling experimental evidence exists for the involvement of lipid exposed surfaces of TM1, TM4 and/or TM5 at the dimerization/oligomerization interfaces of several 7TMRs. Besides, the interface may depend on additional stabilizing factors such as the coiled-coil interactions reported in the $GABA_B$ receptor and the disulfide bridge interactions in the muscarinic and the other class C receptors (reviewed by (Filizola)). A web service, named G-protein coupled Receptors Interaction Partners (GRIP) that predicts the interfaces for 7TMRs oligomerization is also available at http://grip.cbrc.jp/GRIP/index.html (Nemoto et al., 2009). G protein coupled Receptor Interaction Partners DataBase (GRIPDB) has also been developed, which provides information about 7TMRs oligomerization i.e. experimentalaly indentified 7TMRs oligomers, as well as suggested interfaces for the oligomerization (Nemoto et al., 2011).

3.2 Therapeutic application and drug discovery

7TMRs are one of the most important drug targets in the pharmaceutical industry; approximately 40% of the prescription drugs on the market target 7TMRs, but only 5% of the known 7TMR targets are utilized. Agonists and antagonists of 7TMRs are used in the treatment of diseases of every major organ system including the central nervous system, cardiovascular, respiratory, metabolic and urogenital systems. The most exploited 7TMR drug targets include AT_1 angiotensin, adrenergic, dopamine and serotonin (5-hydroxytryptamine, 5-HT) receptor subtypes (Schoneberg et al., 2004). For instance, antagonists of AT_1 angiotensin II receptors are used to prevent diabetes mellitus-induced renal damage and to treat essential hypertension and congestive heart failure. β-adrenergic receptor antagonists, acting on β_1- and/or β_2-adrenergic receptors, are used in patients with congestive heart failure and to treat hypertension and coronary heart disease, while β_2-adrenergic receptor agonists are used in the treatment of asthma, chronic obstructive pulmonary disease and to delay preterm labor. Dopamine receptor antagonists, primarily acting on D_2 receptors, are utilized in the treatment of schizophrenia, while dopamine receptor agonists (e.g. precursor for dopamine levodopa (L-dopa)) remain the standard for treating Parkinson's disease. Inhibitors of 5-HT uptake, which act as indirect agonists at

various subtypes of 5-HT receptors, are used to treat major depressive disorders (Schoneberg et al., 2004).

The increasing importance of dimerization for 7TMRs naturally suggests its possible relevance to drug discovery. It seems that the inclination to hetero-dimerize is common among the 7TM members and that the tissue-specific expression patterns probably underlay the creation of relevant receptor pairs. However, 7TMRs expression has been shown to be altered in some pathological situations. In support to the latter preeclampsia was the first disorder linked to alteration in the AT_1−bradykinin B_2 receptor hetero-dimerization (AbdAlla et al., 2001). Opioid and dopamine receptor hetero-dimerization has also been comprehensively studied, since their putative ligands are used in pathological conditions such as basal ganglia disorders, schizophrenia, drug addiction and pain. The increase in the dopamine D_1-D_3 hetero-dimer was shown to be involved in L-dopa-induced dyskinesia in patients with Parkinson's disease and the addition of an adenosine A_{2A} receptor antagonist potentiates the anti-parkinsonian effect of L-dopa. Hetero-dimers of glutamate receptors mGluR2 and 5-HT_{2A} have been specifically associated with hallucinogenic responses in schizophrenia. Furthermore, the opioid δ-μ receptor hetero-dimer is a better target than either μ or δ receptors alone, since blockade of the δ receptor decreases tolerance to the analgesic effects of the most used μ receptor agonist, morphine (reviewed by (Ferré & Franco, 2010; Kenakin & Miller, 2010)). These observations would probably led to broaden the therapeutic potential of drug targeting 7TMRs and it is also anticipated that the evolving concepts of 7TMR dimerization will be implemented in the BRET-based drug discovery and development process (reviewed by (Casado et al, 2009)).

4. BRET principle and its application in the field of 7TMRs dimerization

4.1 BRET principle

BRET is a biophysical method that enables monitoring of physical interactions between two proteins fused to BRET donor and acceptor moieties, respectively, dependent on their intermolecular distance (10 – 100 Å) and on relative orientation due to the dipole-dipole nature of the resonance energy transfer mechanism (Zacharias et al., 2000). BRET is a non-radiative energy transfer, occurring between a bioluminescent donor that emits light in the presence of its corresponding substrate and a complementary fluorescent acceptor, which absorbs light at a given wavelength and re-emits light at longer wavelengths. To fulfill the condition for energy transfer, the emission spectrum of the donor must overlap with the excitation spectrum of the acceptor molecule (Zacharias et al., 2000). BRET occurs naturally in some marine species (e.g. in the sea pansy *Renilla reniformis*) and in 1999, Xu et al. (Xu et al., 1999) utilized this approach to study dimerization of the bacterial Kai B clock protein. Since then, several versions of BRET assays have been developed that use different substrates and/or energy donor/acceptor couples. The original BRET[1] technology used the pairing of *Renilla luciferase* (Rluc) as the donor and yellow fluorescent protein (YFP) as the acceptor (Xu et al., 1999; Xu et al., 2003). The addition of coelenterazine h, the natural substrate of *Renilla luciferase* (Rluc), leads to a donor emission of blue light (peak at ~480 nm). When the YFP-tagged acceptor molecule, adapted to this emission wavelength, is in close proximity to the Rluc-tagged donor molecule, excitation of YFP occurs by resonance energy transfer resulting in an acceptor emission of green light (peak at ~530 nm). The substantial overlap in the emission spectra of Rluc and YFP acceptor emission (Stokes shift

only ~50 nm) creates a significant problem that has been overcome in a second generation of BRET assay (BRET^2). In BRET^2 assays, *Renilla luciferase* (Rluc) is used as the donor, the green fluorescent protein (GFP) variant GFP^2 as the acceptor molecule (excitation ~400 nm, emission peak at 510 nm) and the proprietary coelenterazine DeepBlueC™ (also known as coelenterazine 400A) as a substrate. In the presence of DeepBlueC™, Rluc emits light peaking at 395 nm, a wavelength that excites GFP^2 resulting in the emission of green light at 510 nm. This modified BRET pair results in a broader Stokes shift of 115 nm, thus enabling superior separation of donor and acceptor peaks, as well as efficient filtration of the excitation light that it does not come to the detector, thereby enabling detection of the weak fluorescence signal. However, the disadvantage of BRET^2, compared to BRET^1 is the 100-300 times lower intensity of emitted light and a very fast decay of emitted light (Heding, 2004). BRET^2 sensitivity can be improved by the development of suitably sensitive instruments (Heding, 2004) and the use of Rluc mutants with improved quantum efficiency and/or stability (e.g. Rluc8 and Rluc-M) as a donor (De et al., 2007). A third generation BRET assay (BRET^3) has been developed recently and combines Rluc8 with the mutant red fluorescent protein (DsRed2) variant mOrange and the coelenterazine or EnduRen™ as a substrate (De et al., 2007). EnduRen™ is a very stable coelenterazine analogue that enables luminescence measurement for at least 24 hours after substrate addition and was utilized in the extended BRET (eBRET) technology (Pfleger et al., 2006). Therefore, in BRET^3, donor spectrum is the same as in BRET^1, and the red shifted mOrange acceptor signal (emission peak at 564 nm) improves spectral resolution to 85 nm, thereby reducing bleedthrough in the acceptor window. Improved spectral resolution and increased photon intensity allow imaging of protein-protein interactions from intact living cells to small living subjects. Additional optimized donor/acceptor BRET couples that combine Rluc/Rluc8 variant with the yellow fluorescent protein, the YPet variant and the Renilla green fluorescent protein (RGFP) has also been developed (Kamal et al., 2009).

4.2 BRET and 7TMRs dimerization

The use of energy-based techniques such as FRET and BRET has been fundamental for taking the theme of 7TMRs dimerization/oligomerization at the front of 7TMRs research. In 2000, BRET was introduced in the 7TMR field demonstrating β_2-adrenergic receptor (β_2-AR) dimerization (Angers et al., 2000) and since then BRET-based information about 7TMRs homo-/hetero-dimerization is rapidly accumulating (for a recent reviews see (Achour et al., 2011; Ayoub & Pfleger, 2010; Ferré et al., 2009; Ferré & Franco, 2010; Gurevich & Gurevich, 2008a; Gurevich & Gurevich, 2008b; Palczewski, 2010)). As a consequence, knowledge databases have been developed to gather and organize these scattered data and provide researchers with the comprehensive collection of information about 7TMR oligomerization. Existing databases are G protein-coupled receptor oligomer knowledge base (GPCR-OKB) (Skrabanek et al., 2007; Khelashvili et al., 2010) that is freely available at http://www.gpcr-okb.org and G protein-coupled receptor interaction partners database (GRIPDB) (Nemoto et al., 2011) available at http://grip.cbrc.jp/GDB/index.html. By analyzing the data in the GPCR-OKB, we can see that BRET-based approaches were used more often than other experimental approaches such as co-immunoprecipitation, cross-linking, co-expression of fragments or modified protomers, use of dimer specific antibodies, fluorescence resonance energy transfer (FRET) and time resolved FRET to detect oligomerization *in vivo* while in *in vitro* systems others methods still prevail (Table 1). The 7TMR pairs for which functional

evidence was provided *in vivo* by BRET are summarized in Table 2. It should be emphasized that besides the intra-family hetero-dimers, the members from different 7TMR families also form functionally relevant inter-family oligomers (Table 2).

Oligomers (*in vivo*)	**7TMR**	**Family A**	**Family B**	**Family C**	**Family A/C**	**Other**
BRET	18	13	0	1	4	0
Mus musculus	7	5	0	1	1	0
Rattus norvegicus	9	5	0	1	3	0
Homo sapiens	9	8	0	0	1	0
Other methods	11	7	0	1	2	1
Oligomers (*in vitro*)						
BRET	50	40	2	1	6	1
Other methods	192	160	4	13	13	2

Table 1. Comparisons of 7TMRs oligomers identified by BRET *vs.* others methods in different 7TMR families in *in vivo* and *in vitro.* Data source GPCR-OKB (http://www.gpcr-okb.org).

Oligomer name	**Organism**	***In vivo* evidence**	**Potential clinical relevance**
Family A 7TMRs			
Adenosine A1 - Adenosine A2A oligomer (**A1 - A2A**)	*Rattus norvegicus*	evidence for physical association in native tissue or primary cells	
Adenosine A2A - Cannabinoid CB1 oligomer (**A2A - CB1**)	*Homo sapiens, Rattus norvegicus*	evidence for physical association in native tissue or primary cells, identification of a specific functional property in native tissue (brain)	Implicated in Parkinson's disease.
Adenosine A2A - Dopamine D2 oligomer (**A2A - D2**)	*Homo sapiens, Rattus norvegicus*	evidence for physical association in native tissue or primary cells, identification of a specific functional property in native tissue (rat striatum, human striatum)	Implicated in Parkinson's desease, schizophrenia. Level of adenosine is increased in the striatal extracellular fluid in Parkinson's disease.
Adrenergic α_1B - Adrenergic α_1D receptor oligomer (**α_1B - α_1D adrenoreceptor**)	*Homo sapiens, Mus musculus*	evidence for physical association in native tissue or primary cells, identification of a specific functional property in native tissue (brain), use of knockout animals or RNAi technology	The study demonstrated that when the α1B-KO and α1D-KO strains of mice are used in conjunction with antagonists, a different pharmacological situation emerges relative to control (sensitivity to Phenylephrine).

Oligomer name	**Organism**	***In vivo* evidence**	**Potential clinical relevance**
Adrenergic α_2A receptor - Opioid μ receptor oligomer (**α_2A-adrenoreceptor – opioid μ**)	*Homo sapiens*	evidence for physical association in native tissue or primary cells	
Adrenergic β_2 - Prostaglandin EP1 receptor oligomer (**β_2-adrenoreceptor - EP1**)	*Homo sapiens, Mus musculus*	evidence for physical association in native tissue or primary cells, identification of a specific functional property in native tissue (airway smooth muscle)	Implicated in decreasing airway smooth muscle relaxation during asthma.
Cannabinoid CB1 - Dopamine D2 oligomer (**CB1 - D2**)	*Homo sapiens, Rattus norvegicus*	identification of a specific functional property in native tissue	
Chemokine CCR2-CXCR4 receptor oligomer (**CCR2 - CXCR4**)	*Homo sapiens*	identification of a specific functional property in native tissue	
Dopamine D1 - Histamine H3 receptor oligomer (**D1 - H3**)	*Mus musculus*	evidence for physical association in native tissue or primary cells	
Dopamine D1 - Opioid μ receptor oligomer (**D1 – μ**)	*Rattus norvegicus*	evidence for physical association in native tissue or primary cells	
Dopamine D2 - Histamine H3 receptor oligomer (**D2 - H3**)	*Homo sapiens Mus musculus*	evidence for physical association in native tissue or primary cells	
Opioid δ - Opioid κ receptor oligomer (**δ – κ**)	*Mus musculus*	colocalization in spinal cord	tissue-specific agonist for pain
Opioid δ - Opioid μ receptor oligomer (**δ – μ**)	*Mus musculus*	evidence for physical association in native tissue or primary cells, identification of a specific functional property in native tissue	
Family C 7TMRs			
γ-aminobutiric acid GABAb receptor oligomer (**GABAB1 - GABAB2**)	*Rattus norvegicus, Mus musculus*	colocalize in brain	$GABA_{B1}$ agonist Baclofen is an antispasm drug
Family A/C 7TMRs			
Adenosine A2A - Metabotropic glutamate 5 (mGLU 5) oligomer (**A2A - mGLU5**)	*Homo sapiens, Rattus norvegicus*	evidence for physical association in native tissue or primary cells	
Dopamine D2 - Metabotropic glutamate 5 (mGLU 5) oligomer (**D2 - mGLU5**)	*Rattus norvegicus*	evidence for physical association in native tissue or primary cells	

Oligomer name	**Organism**	***In vivo* evidence**	**Potential clinical relevance**
Adenosine A2A - Dopamine D2 - Metabotropic glutamate 5 (mGLU5) oligomer (**A2A - D2 - mGLU5**)	*Rattus norvegicus, Mus musculus*	evidence for physical association in native tissue or primary cells	
Serotonin 5-HT2A receptor oligomer - Metabotropic glutamate 2 (**5-HT2A – mGLU2**)	*Homo sapiens*	evidence for physical association in native tissue or primary cells, identification of a specific functional property in native tissue (brain)	5-HT2A levels increase and mGLU2 levels decrease in schizophrenia

Table 2. Intra- and inter-family oligomers with *in vivo* evidence discovered by BRET method. Data source GPCR-OKB (http://www.gpcr-okb.org).

4.3 Interpretation of BRET results – Possible drawbacks

BRET signal indicates that molecules of the same (or two different) receptors are at maximum distance of 100 Å (that equals 10 nm) or more accurately that the donor and acceptor moieties are within this distance. The efficiency of energy transfer depends on the relative orientation of the donor and acceptor and the distance between them (Zacharias et al., 2000), so that absolute distances can not be measured. Experimentally determined Förster distance R_0 (distance at which the energy transfer efficiency is 50%) for $BRET^1$ and $BRET^2$ is 4.4 nm and 7.5 nm, respectively (Dacres et al., 2010). 7TMR transmembrane core spans ~40 Å across the intracellular surface (Palczewski et al., 2000), which makes BRET suitable to the study of dimerization. However, certain facts need to be considered when interpreting BRET results. Firstly, the size of 27 kDa fluorescent proteins and 34 kDa *Renilla luciferase* is comparable to that of the transmembrane core of 7TMRs (diameter ~40 Å). These proteins are usually attached to the receptor C-terminus, which in different 7TMRs varies in length from 25 to 150 amino acids. Polypeptides of this length in extended conformation can cover 80−480 Å. Thus, a BRET signal indicates that the donor and acceptor moieties are at distance less than 10 nm, which may occur when receptors form structurally defined dimer or when they are far >500 Å apart (reviewed by (Gurevich & Gurevich, 2008a)). The use of acceptor and donor molecules genetically fused to 7TMRs can alter the functionality of the receptor; fusion proteins can also be expressed in the intracellular compartments, thus making difficult to demonstrate that the RET results from a direct interaction of proteins at the cell surface (Ferre & Franco, 2010). The use of fusion proteins can therefore be a major limitation for this application. Secondly, quantitative BRET measurements are limited by the quality of the signal and noise level. Fluorescent proteins and luciferase yield background signals arising from incompletely processed proteins inside the cell and high cell autofluorescence in the spectral region used (Gurevich & Gurevich, 2008a). Thirdly, so called bystander BRET results from frequent encounters between overexpressed receptors and has no physical meaning (Kenworthy & Edidin, 1998; Mercier et al., 2002). BRET assays should therefore be able to discriminate between genuine dimerization compared to random collision due to over-expression. To determine specify of BRET signal the following experiments has been proposed: negative control with a non-interacting receptor or protein, BRET saturation and competition assays and experiments that observe ligand-promoted changes in BRET (Achour et al., 2011; Ayoub

& Pfleger, 2010; Ferre & Franco, 2010). Additionally, interpretation of BRET data also requires quantitative analysis of the results, which was so far done only in a small number of studies (Ayoub et al., 2002; Mercier et al., 2002; Vrecl et al., 2006). The theoretical background of the assays described below provides some guidelines for the appropriate interpretation and quantitative evolution of BRET results.

5. Mathematical models to quantitatively assess the oligomerization state of studied receptors

5.1 Basic assumptions

Bioluminescent resonance energy transfer takes place at 1-10 nm distances between molecules thus allowing study of protein-protein interaction. It is a quite robust tool but still some care should be taken with interpretation of the results. Resonance energy transfer is described by the Förster equation for energy transfer efficiency E (Förster, 1959):

$$E = \frac{R_0^6}{R_0^6 + r^6} \tag{1}$$

where r is a distance between donor and acceptor, Förster radius R_0 depends on spectral overlap and dipole orientations yielding R_0 values of 4.4 nm for BRET1and 7.5 nm for BRET2 (Dacres et al., 2010). E is an important parameter in interpretation of the BRET assays used for oligomerisation studies. If the BRET luminometer is properly calibrated then E can be calculated from the $BRET_{\max}$ signal obtained when all donor molecules are accompanied by acceptor molecules:

$$E = \frac{BRET_{\max}}{BRET_{\max} + 1} \tag{2}$$

Calibration should take into account differences in the detector quantum efficiencies at donor and acceptor emission wavelengths and the proportion of the detected emission spectra of both markers. Knowing a Förster radius for certain type of BRET technology used and energy transfer efficiency E we can estimate the distance between the donor and acceptor marker species in the protein complex.

Calculations in presented BRET assays are derived from Veatch and Stryer article (Veatch & Stryer, 1977) covering FRET experiments with Gramicidin dimers. In FRET experiments the 28 $Q/Q0$ is a measurement parameter representing the ratio between not-transmitted energy Q and total energy Q_0. Vaecht and Stryer equations have been adopted for BRET experiments where we measure the ratio between transmitted T and not-transmitted energy Q:

$$BRET = \frac{T}{Q} = \frac{Q_0}{Q} - 1 \tag{3}$$

Single BRET measurements do not give unambiguous proof that receptors form oligomers because the signal can be a consequence of random collisions. To get better indication of the oligomerisation state several quantitative assays were developed.

5.2 BRET dilution assay

This is a simplest control experiment to check for oligomerisation. Resonant energy transfer takes place if the distance between donor and acceptor molecules is in the range of Förster radius R_0. Molecules can get close enough for BRET also by random collisions (bystander BRET) if their density is high enough (Kenworthy & Edidin, 1998; Mercier et al., 2002). Excluding random collisions there should be no concentration dependence for coupled donor and acceptor molecules. In practice we can approximate the *BRET* signal as:

$$BRET = BRET_0 + k([D] + [A]) \tag{4}$$

where [*D*] and [*A*] are donor and acceptor concentrations. With lowering the concentration of both receptors simultaneously (dilution) the *BRET* signal approaches $BRET_0$ which is the real oligomerisation signal (Fig. 1). Dilution assay is used to set the concentration range for saturation and competition assays (Breit et al., 2004).

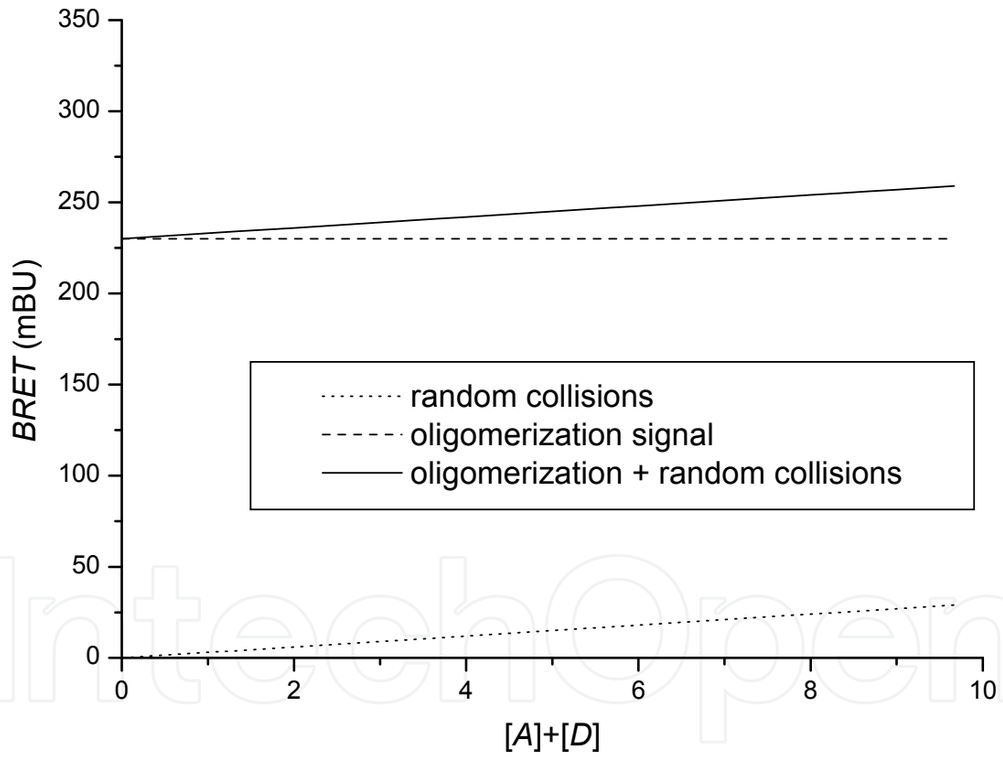

Fig. 1. BRET dilution assay. Theoretical BRET concentration curves for receptors forming monomers or oligomers. A constant ratio between acceptor and donor concentrations should be used.

5.3 BRET saturation assay

Saturation assay involves expressing a constant amount of donor-tagged receptor with an increasing amounts of acceptor-tagged receptor. Theoretically, *BRET* signal should increase with increasing amounts of acceptor until all donor molecules are interacting with acceptor

molecules. Therefore, a saturation level is achieved beyond which a further elevation of the amount of acceptor does not increase the *BRET* signal, thereby reaching a maximal *BRET* level ($BRET_{max}$) (Achour et al., 2011; Ayoub & Pfleger, 2010; Hamdan et al., 2006; Mercier et al., 2002). By using a saturation assay it is possible to obtain the oligomerisation state of homologous receptors. BRET saturation curve is derived from Veatch and Stryer model:

$$BRET = \frac{T}{Q} = \frac{E[AD]}{2[DD] + (1-E)[AD]} \tag{5}$$

where [*AD*] are acceptor-donor and [*DD*] donor-donor dimer concentrations. If all receptors form dimers and association constants are the same for AA, AD and DD we obtain BRET saturation curve for dimers:

$$BRET = \frac{E\frac{[A]}{[D]}}{1 + (1-E)\frac{[A]}{[D]}} \tag{6}$$

For higher oligomers a general BRET saturation curve can be derived (Vrecl et al., 2006):

$$\frac{BRET}{BRET_{\max}} = 1 - \frac{1}{E + (1-E)\left(1 + \frac{[A]}{[D]}\right)^N} \tag{7}$$

where *N*=1 for dimer, *N*=2 for trimer and *N*=3 for tetramer. Theoretical BRET saturation curves are presented in Fig. 2. *BRET* for higher oligomers shows faster saturation. For comparison the monomer *BRET* signal which corresponds to random collisions is presented. If receptor concentration is very high then random collisions can generate saturation curve similar to that of the dimers. Thus a dilution experiment should be done first to distinguish random collisions from the oligomerisation.

In heterologous saturation assay different receptors are used as donors and acceptors. In this case saturation curve is influenced by the affinities for homo-dimer and hetero-dimer formation. In practice we can observe a right-shift of the saturation curve where the association constant for hetero-dimers is smaller than that of the homo-dimers yielding higher $BRET_{50}$ values.

5.4 BRET competition assay

In an attempt to further confirm the existence of oligomer complexes, competition assay can be performed. In this assay the concentration of untagged receptor is increased over a constant concentrations of donor and acceptor tagged receptors (Achour et al., 2011; Vrecl et al., 2006). It is expected that the *BRET* signal would decrease if untagged receptors compete with the tagged receptors for the binding in complexes. Following the Veatch and Stryer approach we obtain *BRET* signal:

$$BRET = \frac{T}{Q} = \frac{E[AD]}{2[DD] + (1-E)[AD] + [CD]} \tag{8}$$

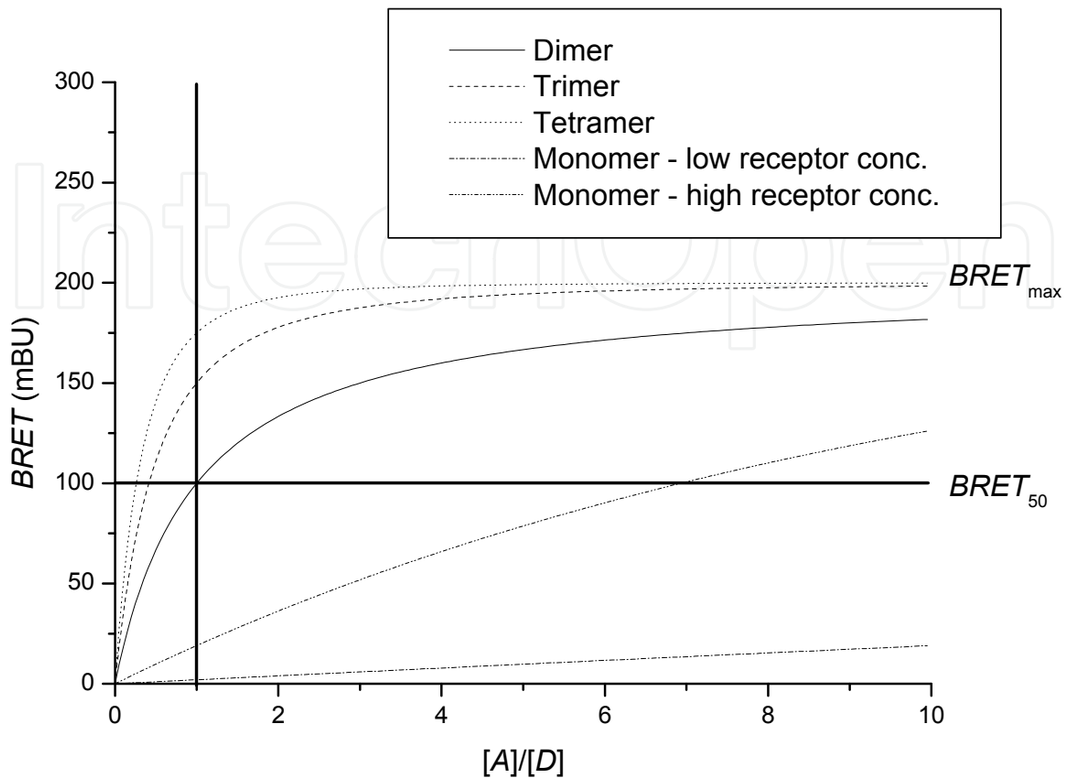

Fig. 2. BRET saturation assay. Theoretical curves for oligomer formation are plotted as a function of ratio of receptors tagged with acceptor [A] and donor [D] molecules. In the case of monomers the BRET signal is created due to random collisions.

where C represents untagged competitor. If all receptors form dimers and association constants are the same for AA, AD, DD, CD, AC and CC dimers we obtain BRET competition curve for dimers:

$$BRET = \frac{E\frac{[A]}{[D]}}{1+(1-E)\frac{[A]}{[D]}+\frac{[C]}{[D]}} \tag{9}$$

Usually in BRET saturation experiments high acceptor to donor concentration ratio is used because the variation in this ratio do not influence the $BRET$ signal as much as for [A]/[D]=1. In general the interaction with the untagged receptors causes the reduction of $BRET$ signal following a hyperbolic curve (Figure 3). We can very well distinguish if the oligomerisation is present, but the exact oligomerisation state is difficult to assess. Competition assay is more suited for the study of hetero-oligomers where different kind of untagged receptor is competing with the homo-oligomers. The saturation curve is shallower if there is a low affinity for hetero-dimer formation compared to homo-dimers

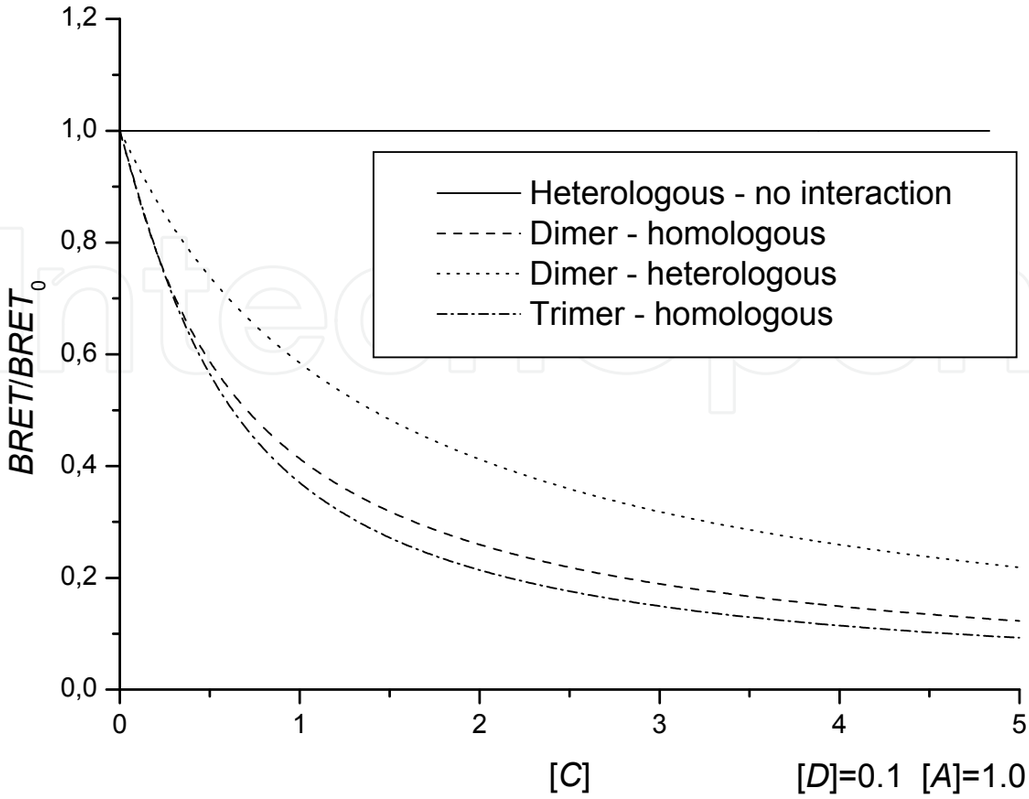

Fig. 3. BRET competition assay. In homologous assay the same receptor is used as a competitor, whereas in heterologous assay different receptor is used. For the latter case a hetero-dimer with lower association constant than that of the homo-dimer is presented.

6. Other BRET-based approaches to identify 7TMR hetero-dimerization

To overcome certain limitations of the classical BRET assays described above, some other BRET-based approaches have been developed to study 7TMR oligomerization/ hetero-dimerization. Sequential-BRET-FRET (SRET) enables identification of oligomers formed by three different proteins. In SRET, the oxidation of the RLuc substrate by an RLuc-fusion protein triggers the excitation of the acceptor GFP^2 by BRET^2 and subsequent energy transfer to the acceptor YFP by FRET. Combination of bimolecular fluorescence complementation (BiFC) and BRET techniques is based on the ability to produce a fluorescent complex from non-fluorescent constituents if a protein-protein interaction occurs. Two receptors are fused at their C-termini with either N-terminal or C-terminal fragments of YFP, respectively, and receptor hetero-dimerization causes YFP reconstitution. Then, if there is hetero-trimerization, BRET can be obtained when the cells also co-express the third receptor fused to Rluc (reviewed by (Ferré & Franco, 2010)). GPCR-Heteromer Identification Technology (GPCR-HIT) utilizes BRET and ligand-dependent recruitment of a 7TMR-specific interaction partners (such as a β-arrestin, PKC or G-protein) to enable 7TMR heteromer discovery and characterization (Mustafa & Pfleger, 2011; See et al., 2011). In this set up, only one receptor subtype is fused to Rluc and the second receptor subtype is untagged. A third protein capable of interacting specifically with one or both receptors in a

ligand-dependent manner is fused to a YFP. Ligand-induced BRET signal indicates that activation of untagged receptor or the heteromer results in recruitment of YFP-tagged protein to the heteromer. Recently developed complemented donor-acceptor resonance energy transfer (CODA-RET) method combines protein complementation with resonance energy transfer to study conformational changes in response to activation of a defined G protein-coupled receptor heteromer. CODA-RET quantify the BRET between a receptor hetero-dimer and a subunit of the heterotrimeric G-protein. It eliminates a contribution from homodimeric signaling and enables analyzing the effect of drugs on a defined 7TMR heterodimer (Urizar et al., 2011).

7. Conclusions

BRET-based techniques are extremely powerful, provided that they are conducted with the appropriate controls and correctly interpreted. Quantitative BRET assays allow us to support the ability of receptor for homo-dimer and hetero-dimer. Homologous saturation assay provide us with the oligomerisation state of receptors. Data interpretation is more difficult for hetero-oligomers and the mixtures of monomer, dimer and higher oligomer populations. For the quantitative approach we also need to know the relative concentrations of all receptors used in the experiment, which can be obtained from radioligand binding, Western blot or ELISA assays.

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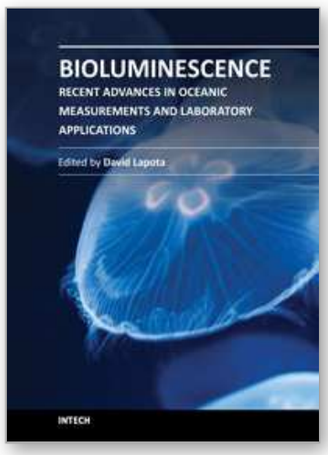

Bioluminescence - Recent Advances in Oceanic Measurements and Laboratory Applications
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