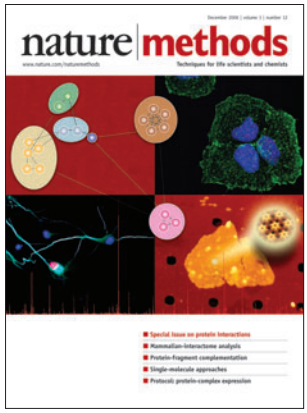
BRET analysis of GPCR oligomerization: newer does not mean better

To the editor: James *et al.*[1] proposed a "rigorous" treatment of bioluminescence resonance energy transfer (BRET) data to distinguish random (nonspecific) from true oligomeric protein interactions. The question is not trivial, and the intention is laudable as BRET is becoming increasingly popular with more than 100 original articles published using the method. James *et al.*, however, dismissed many studies that addressed similar issues, and several points deserve comments.

The authors preface their study by the statement: "Conventional BRET experiments are generally done at maximal expression levels and single acceptor/donor ratios". Although this is true of some studies, such a general statement ignores a large body of work in which these parameters had been taken into account. Expression levels had been monitored in many studies and were found to be within physiological range[2–5], and at least 15 papers had included BRET titration assays in which the donor/acceptor ratio was varied (for example, see refs. 2,4–6). Specificity of BRET signals allowing to distinguish oligomerization from random collisions also has been verified by several authors using BRET competition assays in which the occurrence of BRET between two partners expressed at a given donor/acceptor ratio can be inhibited by expression of the untagged partners but not of an untagged noninteracting protein (for example, see refs. 3,6–8).



James *et al.* propose to differentiate random from true oligomeric interactions based on theoretical considerations summarized in a seminal article by Kenworthy and Edidin[9]. The first approach consists of studying BRET efficiency in experiments in which the acceptor/donor (GFP/luciferase) ratio is varied ('type-1' assay). Random interactions are expected to be less sensitive to the acceptor/donor ratio if the surface density of the acceptor remains low. In multiple previous studies reported in the literature, the change of acceptor/donor ratio was obtained by maintaining the donor concentration fixed and progressively increasing the acceptor; thus, true oligomeric interactions were deduced from the hyperbolic progression of the BRET (for example, see refs. 2,4–6). In their experiments, James *et al.* maintained the total concentration of acceptor and donor constant by inversely changing the concentrations of both donor and acceptor, and used the difference between pseudo- and true-hyperbolic BRET curves to define random collision. Such analysis is technically difficult and complicated by the fact that the efficiency of transfer for random collisions becomes independent of the donor/acceptor ratio only if the acceptor concentration is kept constant (see table 1 in ref. 9).

In 'type-1' assays, James *et al.* also interpreted the lower maximal BRET value as evidence for the lack of dimerization, or equilibrium between dimers and monomers. Such interpretation is very hazardous given that the extent of resonance energy transfer signals vary with the distance between donors and acceptors within a dimer. Thus no direct conclusion can be drawn on the amount of dimers simply based on the maximal BRET signals observed.

In the second set of experiments,'type-2' assays, BRET for true oligomers should be independent of the concentrations of BRET partners at a fixed acceptor/donor ratio. This was previously shown to be the case for class-A G protein–coupled receptors (GPCRs)[2,10,11] and in particular for the ß$_2$AR at receptor concentration below 15 pmol/mg of protein[2]. From their data, James *et al.* concluded otherwise. However, a close examination of the data in Figure 4 reveals that the ß$_2$AR BRET curve has a slope that appears closer to that of the constitutive CTLA-4 dimer than that of the CD2 or CD86 monomers, consistent with the notion that the BRET between ß$_2$AR-luciferase and ß$_2$AR-GFP may reflect constitutive oligomerization. Also, the BRET signal observed for the constitutive dimer CD80 increases more readily with increasing expression levels than that of the ß$_2$AR, further complicating data interpretation. The fact that BRET falls below detection level at low donor/acceptor ratio may reflect lack of detector sensitivity for pairs yielding low BRET signals. Despite these interpretational difficulties, the authors concluded that the entire concept of GPCR oligomerization needs reappraisal.

In their discussion concerning the specificity of the BRET signals observed in previous studies, James *et al.* argued that the GABAb type-2 receptor (GBR2) is a poor choice for a negative control because it can itself dimerize. The reason for using this receptor as a negative control has been precisely its demonstrated ability to dimerize, thus offering a reliable selectivity test using a dimerization-competent receptor. Also, contrary to what the authors implied, GBR2 is not the only negative control that has been used in BRET studies. Several other receptors have been used as negative controls in BRET studies addressing class-A GPCR oligomerization (for example, see refs. 3,12–14).

The observation that in some studies[4,15], ligand binding affects the maximal BRET signal between the proposed protomers of class-A GPCR oligomers, is difficult to reconcile with the implicit conclusion of James *et al.* that the BRET signals observed for class-A GPCRs most likely result from random collisions. In many of the previous studies, the ligand-promoted changes in BRET signal had been interpreted as conformational changes within preexisting dimers that changed the distances between the energy acceptor and donor.

Finally, the notion that family-A GPCRs may form constitutive oligomers is not only based on BRET studies. Many other

biochemical and biophysical approaches support this notion. These include co-immunoprecipitation, various types of FRET, atomic force microscopy, covalent cross-linking, gel filtration, neutron scattering experiments, functional complementation, cell biology studies demonstrating cross-internalization and co-processing of GPCRs as well as binding studies showing positive and negative cooperativity. These approaches, their relative strengths and caveats, including methodological considerations and potential functional outcomes, have recently been reviewed[16,17]. It is therefore premature to dismiss the GPCR oligomer hypothesis based on the interpretations of a single BRET study.

In conclusion, we believe that the results reported in the article by James *et al.* can be interpreted in different ways and that more controls would have been necessary to challenge the multidisciplinary work conducted on this topic by many groups over the past ten years. Clearly, BRET is gaining popularity in assessment of protein-protein interactions in living cells, and additional quantitative approaches will certainly be forthcoming. Maybe more importantly, additional studies performed in native tissues are needed to establish the generality of GPCR dimerization in physiologically relevant systems.

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James and Davis reply: Bouvier *et al.* claim that several theoretical and/or technical limitations undermine our study[1] of class-A G protein-coupled receptor (GPCR) oligomerization using bioluminescence resonance energy transfer (BRET). First, they propose that in order to characterize the dependence of BRET efficiency ($BRET_{eff}$) on acceptor/donor ratio it is not strictly necessary to increase the acceptor/donor ratio by holding the combined amount of acceptor and donor constant and decreasing the amount of donor. They claim that it is equally valid to hold donor levels constant and increase the amount of acceptor. The reason why the acceptor/donor ratio should not be varied in this way is that $BRET_{eff}$ will increase for both randomly interacting monomers and oligomers owing to the concomitant increase in overall protein density, preventing discrimination between the two types of interactions. Second, it is true that in the new method donor and acceptor densities each vary. The important point is that the acceptor density is effectively constant above a certain acceptor/donor ratio threshold, so that, in the case of randomly interacting donors and acceptors, each donor 'experiences' the same acceptor environment as the acceptor/donor ratio increases beyond this threshold. Third, they argue that we gave insufficient consideration to receptor expression level, but we actually show that GPCRs give similarly low acceptor/donor ratio–independent $BRET_{eff}$ irrespective of overall expression from subphysiological amounts to levels 10–100-fold higher than that observed *in vivo*. Fourth, they claim that we were over-reliant on absolute $BRET_{eff}$ levels when in fact we list four criteria that should be used to establish stoichiometry, including absolute $BRET_{eff}$ levels. Fifth, they propose that in 'type-2' BRET experiments (our nomenclature) it is the slope of the line that is important, whereas our control experiments and theory indicate that the defining factor is whether or not the intercept is zero.

Bouvier *et al.* then raise the question of what is to be made of all the biochemical and biophysical data ostensibly supporting class-A GPCR dimerization. We have no direct experience of working with multipass membrane proteins ourselves, but we suspect that these extremely hydrophobic molecules would be prone to artefactual behavior and would exhibit, for example, a tendency to aggregate once extracted from their native membrane environments. The focus of our paper was on the proper implementation of BRET, which, in principle, allows the organizational properties of cell-surface receptors to be examined *in situ* in living cells, free from potential artefacts of this nature. We also note that the controversy surrounding GPCR oligomerization is hardly new[2,3].

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