James and Davis reply: Salahpour and Masri raise several issues[1] concerning our use of 'type-2' bioluminescence resonance energy transfer (BRET) experiments to analyze G protein–coupled receptor (GPCR) oligomerization[2].

The authors present type-2 data for β_2 adrenergic receptor (β_2AR) as well as D1 and D2 dopamine receptors. We restrict our comments to their β_2AR experiments since we have not worked with dopamine receptors. They find that BRET efficiency ($BRET_{eff}$) is independent of expression level, contrary to our observations[2]. The relationship between energy transfer and expression level deviates from pseudolinearity at high expression levels and as overall protein crowding increases[3], as seen in our type-2 data at the highest expression levels[2]. Salahpour and Masri seem to have been unable to reach the low expression levels 48 h after transfection required to observe this effect, given that the lowest level of β_2AR was 100,000 copies per cell, an amount substantially in excess of physiological levels.

The authors are also critical of our use of luminescence detection to follow changes in expression with time, even though it was only this that allowed measurements of $BRET_{eff}$ under conditions for which, for monomeric control proteins, $BRET_{eff}$ was strictly expression dependent[2]. At these low expression levels it is not possible to measure fluorescence accurately enough in plate-based assays to judge protein amounts. We deliberately used a high acceptor/donor ratio (12:1) to limit the effect of any potential variation in the acceptor/donor ratio. Using the analysis shown in figure 3a of our paper[2] for quantitation, the attomolar sensitivity of luminescence detection allowed us to monitor the expression-level dependence of $BRET_{eff}$ at ~1,000 molecules/cell.

Finally, Salahpour and Masri claim that the single value of the soluble GFP-Luc fusion protein fluorescence/luminescence ratio used to determine GFP/luciferase concentration ratios cannot substitute for a standard curve. This was not borne out by our data, which gave very similar values for each construct over many separate experiments. Our experience is that variation in fluorescence/luminescence is the result of slight variations in coelenterazine preparation rather than errors of measurement. Deriving fluorescence/luminescence for each experiment was therefore a more reliable option than using a single standard curve.

As we have emphasized[2], we were not the first to attempt quantitative analyses of BRET data. Previously, however, resonance energy transfer theory was misinterpreted (for example, ref. 4) or applied incorrectly (for example, ref. 5). The only truly novel aspect of our experiments is that we verified our particular implementation of the theory by analyzing a set of very well-characterized monomeric and oligomeric control proteins. Invariably, our type-1 and type-2 protocols correctly identified each of the known monomers and dimers we tested. Using these protocols, class-A GPCRs proved to be exemplars of monomeric behavior as defined by the controls and predicted by the theory. In this context, the technical concerns of Salahpour and Masri do not seem relevant.

John R James & Simon J Davis

Nuffield Department of Clinical Medicine, and Medical Research Council Human Immunology Unit, Weatherall Institute of Molecular Medicine, The University of Oxford, John Radcliffe Hospital, Oxford OX3 9DS, UK.
e-mail: simon.davis@ndm.ox.ac.uk

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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