Supplementary Note 1: Why the dependence of transfer efficiency ($\text{BRET}_{\text{eff}}$) on acceptor/donor ratio is systematically different for randomly interacting proteins (a) versus oligomeric proteins (b), without recourse to theory

BRET relies on non-radiative energy transfer between luciferase (Luc)-coupled donors and green fluorescent protein (GFP)-coupled acceptors. In the illustration below, Luc-coupled donors are represented as blue circles and GFP-coupled acceptors are shown as white (i.e. non-fluorescing) and yellow (i.e. fluorescing) circles. BRET occurs when the acceptor and donor are within 100Å (i.e. a blue and yellow pair is formed). In the upper and lower panels, the yellow circles are pale and bright, respectively, reflecting the $\text{BRET}_{\text{eff}}$ level for each pair ($\text{BRET}_{\text{eff}}$ equals “$x$” for the monomer, and “$y$” for the oligomer; see below).

The two elements of BRET analysis are as follows. First, on a molecule for molecule basis, $\text{BRET}_{\text{eff}}$ for oligomers is higher than that for randomly interacting monomers because the donors and acceptors spend more time within 100Å of each other, and are generally much closer, making energy transfer more efficient (hence the pale and bright yellow circles in the illustration, i.e., $x$ is less than $y$). Overall transfer efficiency will be affinity dependent in the case of oligomers. The cross-sectional area, i.e. the “excluded volume”, of the proteins will also affect transfer efficiency. This differs according to protein class: e.g., type I membrane proteins have a smaller cross-sectional area than multiple pass transmembrane proteins; the smaller the diameter, the closer donors and acceptors get, increasing energy transfer efficiency (for type I membrane proteins, the excluded volume is likely to be set by the
diameters of GFP and luciferase, rather than their transmembrane domains). For this reason it helps greatly to have carefully chosen, protein class-matched controls whose stoichiometry is known.

The second element relies on the prediction, discussed by Kenworthy and Edidin¹, that randomly interacting monomers and oligomers differ fundamentally in their dependence on acceptor/donor ratio ([GFP]/[Luc]). Illustrating this concept, for the examples shown above of a monomeric protein interacting randomly at the cell surface and another protein that forms a constitutive dimer, we start with a field of 20 molecules, with 16 acceptors and 4 donors. The acceptor/donor ratio is increased from 16:4 to 17:3 while keeping the overall density constant (i.e. 20), by removing one donor and replacing it with an acceptor, i.e. by “diluting out” the donor.

The question is: what happens to BRET\text{eff}? A key point is that it is BRET\text{eff}, and not the absolute level of energy transfer, that is important. In the case of the monomer, the initial BRET efficiency is 1x, i.e. each donor is close enough to one acceptor to transfer a fraction of its energy. When the acceptor/donor ratio is increased by replacing one of the donors with an acceptor, the remaining donors remain, on average, close to only one acceptor each, i.e. they continue to experience the same acceptor environment, so BRET\text{eff} remains at the level of 1x. This is only the case at relatively high acceptor/donor ratios, when the density of acceptors is effectively unchanged (in this case it actually changes slightly from 16/20 to 17/20. Empirically, it seems that this situation arises when the acceptor/donor ratio is increased anywhere beyond a threshold of ~2.

For the dimer, because the acceptor/donor ratio is already relatively high (4:1) the field consists mostly of acceptor dimers, but there will be a small number of donor dimers. Overall, the BRET\text{eff} is relatively low (2y/4 = 0.5y). When the acceptor/donor ratio is increased from 16:4 to 17:3 by replacing the donor with the acceptor, however, this doubles BRET\text{eff} (to 3y/3 = 1y). Under conditions of essentially constant density and increasing acceptor/donor ratio, therefore, there is a non-linear (i.e. hyperbolic) increase in BRET\text{eff}. The overall BRET\text{eff} level, and the dependence of BRET\text{eff} on acceptor/donor ratio at constant density or on expression level at constant acceptor/donor ratio (see the main text), allow these two types of interactions to be distinguished.