Hill Stephen John (Orcid ID: 0000-0002-4424-239X)
Kilpatrick Laura E (Orcid ID: 0000-0001-6331-5606)

Kinetic analysis of fluorescent ligand binding to cell surface receptors: Insights into conformational changes and allosterism in living cells.

Stephen J. Hill[1,2,*] and Laura E Kilpatrick[2,3]

[1] Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, University of Nottingham, Nottingham NG7 2UH, UK
[2] Centre of Membrane Proteins and Receptors, University of Birmingham and Nottingham, The Midlands, UK
[3] Division of Bimolecular Science and Medicinal Chemistry, School of Pharmacy, Biodiscovery Institute, University of Nottingham, NG7 2RD, UK

*Corresponding authors:
Stephen J. Hill, Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, University of Nottingham, Nottingham NG7 2UH, UK: stephen.hill@nottingham.ac.uk

Key words: Ligand-binding, kinetics, allosterism, conformational changes, receptors, fluorescent ligands.

Short title: Ligand-binding kinetics and conformation change.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.16185

Abbreviations.

BRET, Bioluminescence Resonance Energy Transfer
NRP1, Neuropilin-1
TMR, Tetramethylrhodamine
VEGF, Vascular Endothelial Growth Factor
K_D, equilibrium dissociation constant
k_{on}, on rate constant
k_{off}, off rate constant
RAMP, receptor activity-modifying protein
GPCR, G protein-coupled receptor
RTK, receptor tyrosine kinase
TR-FRET, time-resolved Förster resonance energy transfer
NanoLuc, nanoluciferase
NECA, N-ethylcarboximidoadenosine
XAC, xanthine amine congener
PD81,723, (2-amino- 4,5-dimethyl-3-thienyl)(3-(trifluoromethyl)phenyl)-methanone
IL-23, interleukin-23
NanoBiT, NanoLuc Binary Technology

Abstract.

Equilibrium binding assays are one of the mainstays of current drug discovery efforts to evaluate the interaction of drugs with receptors in membranes and intact cells. However, in recent years there has been increased focus on the kinetics of the drug-receptor interaction to gain insight into the lifetime of drug-receptor complexes and the rate of association of a ligand with its receptor. Furthermore, drugs that act on topically distinct sites (allosteric) from those occupied by the endogenous ligand (orthosteric site) can induce conformational changes in the orthosteric binding site leading to changes in the association and/or dissociation rate constants of orthosteric ligands. Conformational changes in the orthosteric ligand binding site can also be induced through interaction with neighbouring accessory proteins and receptor homo- and hetero-dimerization. In this review, we provide an overview of the use of fluorescent ligand technologies to interrogate ligand-receptor kinetics in living cells and the novel insights that they can provide into the conformational changes induced by drugs acting on a variety of cell surface receptors including G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and cytokine receptors.

1. Introduction.

Equilibrium binding assays are one of the mainstays of current drug discovery efforts to evaluate the interaction of drugs with receptors in membranes and intact cells. An important measure is the equilibrium dissociation constant (K_D) that describes the concentration of a drug that is required to occupy 50% of its target receptor at equilibrium. However, in recent years there has been increased focus on the kinetics of the drug-receptor interaction to gain insight into the lifetime of drug-receptor complexes (provided by the residence time, which is the reciprocal of the off rate constant k_{off}) and the rate of association of a ligand with its receptor (provided by the on rate constant, k_{on}) (Copeland, 2016; Schuetz et al., 2017; Sykes et al 2019; Ijzerman & Guo, 2019). Furthermore, drugs that act on sites on the target receptor that are topically distinct (allosteric) from those occupied by the endogenous ligand (orthosteric site) can modulate the binding of orthosteric ligands by changing their association or dissociation rate constants, as a consequence of the conformational changes they induce (**Figure 1a**; Kostenis et al., 1996; Molderings et al., 2000; Gao et al., 2001, Guo et al., 2014; van der Westhizen et al., 2015; Sykes et al., 2019; Cooper et al., 2019; Draper-Joyce et al., 2021).

In addition to the influence of small molecule allosteric ligands on orthosteric ligand binding, conformational changes in the orthosteric ligand binding site can also be regulated through interaction with neighbouring accessory proteins and receptor homo- and hetero-dimerization (**Figure 1b**; Sykes et al., 2019). For example, heterodimers between the calcitonin receptor and one of three receptor activity-modifying proteins (RAMPs) can yield very distinct receptor properties (Hay et al., 2015; Cao et al., 2022). Co-operative interactions across homodimer interfaces can also lead to dramatic effects on the dissociation rate of orthosteric ligands (May et al., 2011; Gherbi et al., 2015). As a consequence of these influences, analysis of the kinetics of ligand-binding interactions can provide important insights into the conformational changes induced in the target receptor by ligands, allosteric modulators and protein-protein interactions (**Figure 1**). In this review, we provide an overview of the use of fluorescent ligand technologies to interrogate ligand-receptor interactions in living cells and the novel insights that they can provide into the conformational changes induced by drugs acting on a variety of cell surface receptors including G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and cytokine receptors.

2. Fluorescent ligands

Fluorescent ligands are beginning to emerge as the labelled probe of choice for kinetic and equilibrium binding measurements in both intact cells and membrane preparations (Vernall et

al., 2014; Peach et al., 2018; Peach et al., 2021; Stoddart et al., 2015; Stoddart et al., 2016; Sykes & Charlton, 2018; Schiele et al., 2015; Suchankova et al., 2021; Borgarelli et al., 2021; Lay et al., 2022; Gratz et al., 2022; Toy et al., 2022; Honsou et al., 2015; Honsou et al., 2018). This is primarily because of their ability to monitor ligand-binding in real-time at the single cell level which is a distinct advantage over radioligand approaches (Peach et al., 2018; Bouzo-Lorenzo et al., 2019; Stoddart et al., 2018b; Wesslowski et al., 2020). However, the important concepts developed using radioligand binding studies still apply to fluorescent ligand binding approaches and it is worth noting that the addition of a radiolabel produces a much smaller change in the molecular structure of the original molecule than is achieved by the addition of a fluorophore.

An important feature of small molecule fluorescent probes for GPCRs is that the addition of the fluorophore and linker to the pharmacophore can dramatically increase the molecular weight of the final compound and alter its physicochemical properties (particularly hydrophobicity and/or lipophilicity) and molecular pharmacology (Baker et al., 2010; Vernall et al., 2014; Stoddart et al 2016; Kok et al., 2022; Comeo et al., 2020; Comeo et al., 2021). The choice of the fluorophore can also alter the pharmacological properties of the original ligand suggesting that it may contribute to the final binding affinity (Baker et al 2010; Comeo et al., 2020; Comeo et al., 2021). It is therefore very important to fully characterise the pharmacology of small molecular weight fluorescent probes (i.e., treat them as new chemical entities). Where the orthosteric ligand is of much higher molecular weight, the labelling strategies are more straightforward and success has also been achieved with chemokines (White et al., 2020), growth factors (Comez et al., 2022; Kilpatrick et al 2017), cytokines (Lay et al., 2022), cyclic peptides (Lay et al., 2023a), eGFP-Wnt-3a (Wesslowski et al., 2020; Gratz et al., 2023) and receptor-targeted nanobodies (Comez et al., 2022; Van den Bor et al., 2023).

Direct measurement of the binding of fluorescent ligands can be achieved using confocal microscopy (May et al., 2010; Gherbi et al., 2015; Comeo et al 2021, 2022). However, its success in kinetic studies depends on a low level of non-specific binding and is most effective with fluorophores (e.g. BODIPY 630/650) that are heavily quenched in an aqueous environment (such that free dye is not detected within the confocal volume). BODIPY dyes have the advantage that they are much brighter when bound to a receptor on the cell membrane, and where the BODIPY moiety can partition into the membrane environment. BODIPY dyes are, however, highly lipophilic and this can lead to their non-specific accumulation in cell membranes and intracellular environments producing an increase in non-specific binding and higher background signals (Rose et al., 2012; Arruda et al., 2017). For example, BODIPY630-650-mepyramine suffered from a high intracellular uptake that

compromised confocal imaging studies with this histamine H_1-receptor fluorescent ligand (Rose et al., 2012). Nevertheless, single molecular approaches such as fluorescence correlation spectroscopy have been able to use this ligand to study ligand-binding properties successfully in small microdomains of the plasma membrane (Rose et al., 2012). Thus, for higher throughput applications, even confocal imaging multi-well plate readers may not have sufficient signal to noise ratios for reliable measurements (Arruda et al., 2017).

The emergence of resonance energy transfer methods such as time-resolved Förster resonance energy transfer (TR-FRET) or bioluminescence energy transfer (BRET) using fluorescent ligands has revolutionised our ability to undertake kinetic-based ligand-binding studies (Schiele et al., 2015; Stoddart et al., 2015; Stoddart et al., 2018; Sykes et al, 2017; Sykes & Charlton, 2018). This is because energy transfer is only detected when the energy donor (i.e. the fluorescent or bioluminescent protein) is in close proximity (<10nm) to the acceptor fluorescent ligand. Consequently, there is no need to wash away the fluorescent ligand and the measurements can be made in real-time. TR-FRET normally uses self-labelling fusion proteins such as SNAP or HaloTag (Keppler et al., 2003; Los et al., 2008; Comeo et al, 2020 Sykes & Charlton, 2018) attached to the N-terminus of a receptor (e.g. GPCR or RTK) which can be covalently labelled *in vivo* with a substrate carrying a lanthanine cryptate (e.g. terbium). TR-FRET can then occur following energy transfer from the terbium donor to the fluorescent ligand if the fluorescent ligand is bound to a site on the receptor that is in very close proximity (<10nm) to the SNAP or HaloTag donor on the N-terminus of the receptor (Schiele et al 2015; Sykes & Charlton, 2018; Comeo et al., 2020). The time-resolved aspect of TR-FRET is particularly helpful since the fluorescence of terbium is long-lived and this allows a time delay of 50-150 μsec to be made between excitation of the terbium fluorophore and measurement of the emission from the acceptor fluorescent ligand. This can markedly reduce the background autofluorescence observed in live cell and membrane experiments (Sykes et al., 2019). In the case of BRET, the receptor is tagged (usually on the N-terminus) with the small luminescent protein nanoluciferase (NanoLuc) which in the presence of its substrate furimazine can produce a very bright bioluminescence (Hall et al., 2012). This can then transfer energy via resonance energy transfer to a fluorescent ligand bound to the receptor at a site in close proximity to the N-terminus (<10nm) (Stoddart et al., 2015; Kilpatrick et al., 2017; Peach et al., 2018; 2021; Goulding et al., 2021; Lay et al., 2022).

3. Ligand-binding kinetics.

As mentioned above, an important measure of equilibrium binding is the dissociation constant (K_D) that describes the concentration of a drug that is required to occupy 50% of its target receptor at equilibrium. This can be altered by allosteric modulators or the cooperative action

of neighbouring proteins via protein-protein complexes involving proteins such as RAMPs or the product of receptor dimerization (**Figure 1a,b**; Cooper et al., 2019; May et al., 2011; Gherbi et al., 2015; Hay et al., 2015; Cao et al., 2022). A change in ligand binding affinity can be readily detected in saturation binding experiments (**Figure 1c**) whereas the impact on ligand-receptor dissociation kinetics can clearly be observed if the dissociation rate constant (k_{off}) is altered by protein-protein interactions or the action of allosteric modulators (**Figure 1d**). For example, the positive allosteric modulator VCP171 can induce a marked increase in the pK_D values of a fluorescent analogue of the agonist N-ethylcarboximidoadenosine (NECA; BY630-X-AAG-ABEA) binding to the human adenosine A_1-receptor (Cooper et al., 2019). Positive allosteric effects can also be detected as a decrease in the off rate of a fluorescent agonist. Thus, when the dissociation of the fluorescent adenosine analogue ABA-X-BY630 was monitored at the single cell level in cells expressing the human adenosine A_1-receptor, it was significantly faster in the absence (k_{off} 1.95 min^{-1}) compared with the presence of the allosteric enhancer (2-amino- 4,5-dimethyl-3-thienyl)(3-(trifluoromethyl)phenyl)-methanone (PD81,723; 10 μM; k_{off} 0.8 min^{-1}; May et al., 2010).

Association kinetic assays using two or more concentrations of fluorescent ligand are the simplest kinetic measurements to make when using TR-FRET or BRET experimental configurations (**Figure 2**; Sykes et al., 2019). The relationship between the observed association rate constant (k_{obs}) and the equilibrium rate constants k_{on} and k_{off} is given by the following equation:

$$k_{obs} = k_{on}\, x\, [Label] + k_{off}$$

Where [Label] represents the concentration of fluorescent probe used. If more than one concentration of fluorescent ligand is employed (and parallel conditions are run to assess the kinetics of the non-specific component of binding at each fluorescent ligand concentration) then k_{on} and k_{off} can be shared between the non-linear regression fits of specific binding to allow these constants to be measured directly from the association kinetic experiments. We have successfully used this approach in NanoBRET and TR-FRET experiments to evaluate the k_{on} and k_{off} rate constants for a wide range of different fluorescent ligands for both GPCRs and RTKs (Stoddart et al., 2018; Bouzo-Lorenzo et al., 2019; Peach et al., 2019; Comeo et al., 2020; Kok et al., 2022). It is worth emphasising that this analysis assumes a simple mass action equilibrium between the fluorescent ligand and its target receptor. It is therefore important to check that there is no cooperativity (positive or negative) occurring at higher fluorescent ligand concentrations across dimer interfaces that can affect this simple analysis

(e.g. May et al., 2011; Gherbi et al., 2015; see also section 4 below). For example, we have successfully prepared a fluorescent version of vascular endothelial growth factor 121a ($VEGF_{121}a$) with a single tetramethylrhodamine (TMR) fluorophore added to each of the antiparallel $VEGFR_{121a}$-TMR homodimer components (Kilpatrick et al., 2017; Peach et al., 2018a, 2019, 2021). This molecule binds to the Ig-like domains D2 and D3 of VEGFR2 with a stoichiometry of one $VEGF_{121}a$-TMR dimer across a VEGFR2 homodimer (Peach et al., 2018b). A simple analysis of the binding kinetics of $VEGF_{121}a$-TMR to membrane preparations obtained from HEK293 cells expressing human NanoLuc-tagged VEGFR2 receptors and assuming a simple mass action equilibrium yields k_{on} and k_{off} estimates of 5.13×10^6 $min^{-1}M^{-1}$ and 0.019 min^{-1} respectively and a kinetic K_D value of 3.85nM (**Figure 3a**; Peach et al., 2019).

However, it is known that VEGF can induce homodimerization of VEGFR2 (Kilpatrick et al., 2019) and so it is very likely that the binding of $VEGF_{121}a$-TMR can occur in a stepwise fashion as depicted in **Figure 3b**. As a consequence, the binding of $VEGF_{121}a$-TMR can be described as a two-step process where binding of the growth factor first occurs to one molecule of VEGFR2 and is then followed by recruitment of a second VEGFR2 molecule to the complex as the $VEGF_{121}a$-TMR homodimer engages the binding site on the second VEGFR2 monomer bridging across the VEGFR2 dimer (**Figure 3b**) (White et al., 2022). The first phase of binding to one VEGFR monomer can be described by simple mass action kinetics involving k_{on} and k_{off} (**Figure 3b**). However, the second phase involving ligand-induced dimerization requires the inclusion of cooperativity rate constants (θ_+ and θ_-) to describe binding to the second VEGFR2 protomer (**Figure 3b**). White et al (2022) used this approach to fit the data set from Peach et al., (2019) to this model. This analysis revealed k_{on} and k_{off} rate constants (**Figure 3b**) of a similar order to those described by Peach et al (2019) and indicated that the second phase of binding was an extremely rapid step and acted to increase the binding affinity from 26.3nM (for the monomer) to 1.64 nM (for the homodimer).

The other approach to monitoring ligand dissociation kinetics is to allow the binding of the fluorescent to reach equilibrium and then to either wash away the label using infinite dilution approaches or to add an excess of a non-fluorescent or non-radioactive ligand that binds to the same orthosteric site as the label (**Figure 4**). Using this approach, the kinetics of the dissociation phase of ligand-receptor interactions can be monitored (**Figure 4**). The infinite dilution approach is quite difficult in a plate-reader configuration because of the excessive wash steps required to prevent rebinding of the label (to neighbouring receptors) as it dissociates from the receptor (Sykes et al., 2019). However, success has been achieved in imaging set-ups where it is possible to perfuse the imaging cell rapidly with buffer (May et al., 2010). Thus, we have been able to perfuse an imaging cell with buffer at flow rates of up to

20 ml.min^{-1} without losing focus whilst acquiring images on a confocal microscope (May et al., 2010). This allowed rapid addition and removal of drugs within a timescale of seconds (May et al., 2010). Using this approach, we were able to monitor the on (k_{on}) and off (k_{off}) rates of a fluorescent adenosine analogue in real time to individual cells (in the field of view) expressing either the A_1 or A_3 adenosine receptors (May et al., 2010).

In a plate reader configuration, the approach of choice to monitor ligand dissociation kinetics is to add an excess of a non-fluorescent that binds to the same orthosteric site as the label (**Figure 4**). Provided that ligand-binding adheres to simple mass action kinetics, this approach avoids issues with re-binding (Sykes et al., 2019) and allows direct measurement of k_{off}. Allosteric ligands can also be applied during the dissociation phase of the experiment (infinite dilution or addition of excess orthosteric ligand) to evaluate whether they have a positive (reduced k_{off}) or negative (increased k_{off}) allosteric effect on fluorescent ligand dissociation (**Figure 4**).

4. Impact of receptor homodimerization on ligand-binding properties – insights from ligand dissociation studies.

In addition to the impact of allosteric ligands on measured k_{off} rate constants, orthosteric ligands can have an unexpected effect on this parameter if the target receptor involved forms homodimers (May et al., 2011). Thus, in the case of the adenosine A_3-receptor, orthosteric ligands such as adenosine, NECA and the antagonist xanthine amine congener (XAC) can increase the measured k_{off} rate constant for the fluorescent adenosine analogue ABA-X-BY630 by an order of magnitude (May et al., 2011). Furthermore, if the wild-type A_3 receptor is co-expressed with a non-binding N250A A_3-receptor mutant (in order to reduce the number of homodimers containing two available orthosteric binding sites), this effect is reduced (May et al., 2011). The data obtained were consistent with orthosteric ligands producing negative allosteric effects across adenosine A_3-receptor homodimer interfaces (**Figure 5**). In the scheme shown in **Figure 5**, the receptor is assumed to exist as a homodimer in which each receptor protomer can bind drug A or B. Binding of A or B to one protomer of the dimeric structure is described by the simple equilibrium binding constants K_a and K_b respectively. However, once one protomer is occupied by drug, the subsequent binding of a drug molecule to the second orthosteric site on the dimer is influenced by the cooperativity factors α, β and γ. The fitted values for these cooperativity factors for the fluorescent adenosine analogue ABA-X-BY630, NECA and XAC obtained in the study of May et al (2011) are provided in **Figure 5**. For ABA-X-BY630 (as drug A) the α factor of <0.01 indicates that the binding of ABA-X-BY630 requires very much higher concentrations

of ligand to achieve occupancy of the second protomer site due to substantial negative cooperativity across the dimer interface. Similarly, addition of NECA or XAC during the dissociation phase following washout of fluorescent ligand when the fluorescent ligand is only occupying one of the protomers (i.e. AR-R) will be markedly affected by the substantial negative cooperativity (γ = 0.13 or 0.07) caused by NECA or XAC occupying the second protomer binding site (AR-RB) (May et al., 2011). We have observed similar negative cooperative interactions across β_1-adrenoceptor homodimers (Gherbi et al., 2015). Interestingly, the β_1-adrenoceptor has been reported to mediate signalling effects via both a high affinity catecholamine site and a low affinity CGP 12177 site (Kaumann and Molenaar, 2008; Baker et al., 2003). It is likely that the low affinity site is a consequence of negative cooperativity across the dimer interface (Gherbi et al., 2015 Feuerstein and Schlicker, 2021).

From a kinetic perspective, it is worth pointing out that the cooperativity factors α, β and γ shown in Figure 5 will have both association and dissociation rate constants associated with them (i.e. α_+/α_-, β_+/β_- and γ_+/γ_-) in a similar way to the cooperativity factor θ (θ_+ and θ_-) shown in Figure 3(b). These can have different contributions to the overall allosteric effect as shown using various mathematical models for different allosteric mechanisms of action (Diaz et al., 2023; White et al, 2022).

5. Heterodimerization and impact on ligand binding affinity.

Heterodimerization is an obvious way in which neighbouring proteins can also exert allosteric effects on the binding of fluorescent probes to the orthosteric binding site of the target receptor (**Figure 1b**). As noted above, heterodimers between the calcitonin receptor and one of three receptor activity-modifying proteins (RAMPs) can yield very distinct receptor properties (Hay et al., 2015; Cao et al., 2022). Receptor-receptor interactions can have a similar effect. For example, the interleukin-23 (IL-23) cytokine binds to a heterodimeric receptor made up of two subunits - IL12Rβ1 and IL23R (Parham et al., 2002; Lay et al., 2022). TMR-labelled IL-23 (IL23-TMR) can bind to a NanoLuc-tagged IL23R with a K_D of 222 nM and to a NanoLuc-tagged IL12Rβ1 with a K_D of 30 nM (Lay et al., 2022). However, when NanoLuc-IL23R is co-expressed alongside untagged IL12Rβ1 the affinity of IL23-TMR is increased by nearly four orders of magnitude to 27 pM (Lay et al., 2022). Thus, the heterodimeric IL23R-IL12Rβ1 complex has a dramatic effect on its affinity for its native cytokine when compared to either receptor alone. Studies on purified truncated receptor proteins have suggested that the IL-23 receptor may be activated following ligand-induced dimerization where IL-23 first binds to IL23R and then recruits IL12Rβ1 to the IL23R-IL12Rβ1 oligomeric complex (Bloch et al., 2018). i.e. In a similar fashion to that described

for VEGFR2 (**Figure 3b** above). However, an alternative explanation deduced from recombinant cellular systems is that IL23R is activated via a conformational change within pre-associated complexes (Sivanesan et al., 2016; Lay et al., 2022). Further insight into this latter mechanism has been provided using NanoLuc Binary Technology (NanoBiT) (Dixon et al., 2016). The NanoBiT methodology uses fragments of NanoLuc which is split into a large (18 kDa) fragment (LgBiT) and smaller (1.3 kDa) high affinity (HiBiT) or low affinity (SmBiT) peptides (Dixon et al., 2016). When HiBiT and LgBiT fusions of NanoBiT fragments are attached to the N-termini of IL23R and IL12Rβ1 respectively, the association of the receptor proteins can be monitored by following the re-complementation of the full length NanoLuc bioluminescent protein (Dale et al., 2019; Lay et al., 2023b). Using this approach, we were able to show that IL23-TMR was able to bind with picomolar affinity to pre-formed HiBiT-IL23R-LgBiT-IL12Rβ1 heteromeric complexes without any change in the extent of heteromer formation induced by the cytokine (Lay et al., 2023b). A similar NanoBiT strategy has been used to investigate the interaction of VEGFR2 with its co-receptor neuropilin 1 (NRP1) (Peach et al., 2021). This has been achieved by using NanoBiT technology in combination with NanoBRET to monitor the kinetics of the binding of fluorescent VEGF-A analogues to defined heteromeric VEGFR2-NRP1 complexes (Peach et al., 2021).

6. Conformational sensors.

The attachment of a tag to an extracellular domain of a cell surface receptor also provides the potential to monitor conformational changes induced by agonists, antagonists and allosteric modulators. We became aware of this in early studies to investigate NanoBRET ligand binding to the chemokine receptor CXCR4 using CRISPR/Cas9 genome editing to attach a HiBiT tag to the N-terminus of the endogenous CXCR4 receptor (White et al., 2020). This allowed binding of a fluorescent version of the chemokine CXCL12 (CXCL12-AF647) to be measured at endogenous receptor expression levels that were only 1-2% of the levels normally achieved in transfected cells (White et al., 2020). In this approach, purified LgBiT was added to the extracellular medium of cells to reconstitute the full length NanoLuc in association with cell surface receptors expressing the HiBiT-tagged CXCR4 receptors (White et al., 2020). Because LgBiT is cell impermeable, reconstitution between LgBiT and HiBiT on the N-terminus of CXCR4 is restricted to receptors at the plasma membrane. When cells are treated with the agonist CXCL12, internalisation of CXCR4 can be followed as a loss of receptor associated NanoLuc luminescence at the cell surface (White et al., 2020). However, surprisingly, the negative allosteric modulators IT1t and AMD3100 caused an increase in NanoLuc luminescence in both CRISPR/Cas9 edited HiBiT-CXCR4 expressing HEK 293 cells and in cells exogenously transfected with HiBiT-

CXCR4 (White et al., 2020). This was not, however, due to appearance of new receptors at the cell surface since the same phenomenon could be detected in isolated membrane preparations (White et al., 2020). The data obtained were most consistent with a conformational change in CXCR4 induced by IT1t and AMD3100 which reduced steric hindrance and facilitated the engagement of the N-terminally attached HiBiT with the free LgBiT in the extracellular medium (White et al., 2020). This conformational change can be followed in real time in membrane preparation following addition of the two negative allosteric modulators (**Figure 6**).

Another approach to add a conformation-sensing tag to a receptor is to use the antigen-binding immunoglobulin (Ig) variable region (nanobody) of a heavy-chain antibody derived from the camelid family (Comez et al., 2022). We have recently investigated the binding mode of two fluorescently-tagged nanobodies that bind to the epidermal growth factor receptor (EGFR) (Comez et al., 2022). In this study, we investigated the binding of fluorescent analogues of Q86 and Q44 to NanoLuc-tagged EGFR. Q44 is a nanobody that binds to a similar site to EGF on EGFR (domains I and III) whereas Q86 (also known as EgB4) is an EGFR nanobody that does not compete with EGF binding and does not activate the receptor (Comez et al., 2022). EGF did not inhibit the binding of fluorescent Q86 (Q86c-HL488) but instead caused a marked increase in the NanoBRET signal consistent with a conformational change altering the efficiency of resonance energy transfer between the NanoLuc on the N-termini of EGFR and the fluorophore on the Q86 molecule bound to EGFR (Comez et al., 2022*).* This is most likely due to the extended conformation of EGFR induced by EGF (**Figure 7a** inset) leading to exposure of the dimerization interfaces of domain II of the receptor and subsequent EGFR homodimerization (Dawson et al., 2005; Bessman et al., 2014; Freed et al., 2017) (**Figure 7a**). This conformational change is also consistent with the recent X-ray crystal structures of Q86 (EgB4) alone and bound to the full extracellular EGFR-EGF complex in its extended active conformation (Zeronian et al., 2022). Real-time NanoBRET experiments allow the time course of the binding of Q86c-HL488 to be followed as well as the conformational change induced by subsequent addition of different concentrations of EGF (**Figure 7a**; Comez et al., 2022). Furthermore, the ability of different EGFR agonists to induce this conformational change can also be quantified (**Figure 7b**).

7. Conclusions and future directions.

In conclusion, analysis of the impact on ligand-binding kinetics of allosteric ligands and cooperative interactions across protein-protein interfaces can provide important information on the conformational changes that they induce. The fact that these interactions can be

monitored in living cells at the single cell level provides a powerful approach to the study of cooperative interaction across protein-protein interfaces in real time. In this review we have summarised some of the approaches using fluorescent ligands, fluorescent nanobodies and resonance energy transfer approaches. However, in many of these instances, the experimental approach requires recombinant transfection techniques to introduce tagged-receptors (e.g. SNAP-tag, HaloTag or NanoLuc) into model cell systems. This invariably leads to expression levels that are one or two orders of magnitude greater than normal physiological levels. However, more recently we and others have been using CRISPR/Cas9 genome editing to introduce tags onto the N-terminus of endogenous receptors (White et al., 2019; White et al., 2020; Soave et al., 2021; Goulding et al., 2021a; Goulding et al., 2021b; Gratz et al., 2023). These approaches provide a very sensitive approach to study ligand-receptor interactions at extremely low expression levels using NanoBRET (White et al., 2019; White et al., 2020; Goulding et al., 2021a) or biophysical techniques with single molecule sensitivity such as fluorescence correlation spectroscopy (Goulding et al, 2021b) and pulsed-interleaved fluorescent cross-correlation spectroscopy (Endres et al., 2013).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

ACKNOWLEDGEMENTS

This work in the authors' laboratories is funded by funded by the Medical Research Council (grant numbers MR/N020081/1 and MR/W016176/1). L.E.K is supported by a University of Nottingham Anne McLaren Fellowship.

References.

Alexander S.P.H., Kelly E., Mathie A., Peters J.A., Veale E.L., Armstrong J.F., Faccenda E., Harding S.D., Pawson A.J., & Southan C. (2021) The concise guide to pharmacology 2021/22: Introduction and other protein targets. Br J Pharmacol. 178 S1-S26.

Arruda, M. A., Stoddart, L. A., Gherbi, K., Briddon, S. J., Kellam, B., & Hill, S. J. (2017). A Non-imaging High Throughput Approach to Chemical Library Screening at the Unmodified Adenosine-A_3 Receptor in Living Cells. Frontiers in Pharmacology, *8*, 908.

Baker JG, Hall IP, Hill SJ. (2003) Agonist actions of "beta-blockers" provide evidence for two agonist activation sites or conformations of the human beta1-adrenoceptor. Mol Pharmacol. 63, 1312-21.

Baker JG, Middleton R, Adams L, May LT, Briddon SJ, Kellam B, Hill SJ. (2010) Influence of fluorophore and linker composition on the pharmacology of fluorescent adenosine A1 receptor ligands. Br J Pharmacol. 159, 772-86.

Bessman NJ, Bagchi A, Ferguson KM, Lemmon MA. (2014) Complex relationship between ligand binding and dimerization in the epidermal growth factor receptor. Cell Rep (9, 1306-17.

Bloch, Y., Bouchareychas, L., Merceron, R., Składanowska, K., Bossche, L. Van den, Detry, S., et al. (2018). Structural Activation of Pro-inflammatory Human Cytokine IL-23 by Cognate IL-23 Receptor Enables Recruitment of the Shared Receptor IL-12Rβ1. Immunity *48*, 45–58.

Borgarelli, C., Klingl, Y. E., Escamilla-Ayala, A., Munck, S., Van Den Bosch, L., De Borggraeve, W. M., & Ismalaj, E. (2021). Lighting Up the Plasma Membrane: Development and Applications of Fluorescent Ligands for Transmembrane Proteins. Chemistry, *27*, 8605–8641.

Bouzo-Lorenzo M, Stoddart LA, Xia L, IJzerman AP, Heitman LH, Briddon SJ, Hill SJ. (2019) A live cell NanoBRET binding assay allows the study of ligand-binding kinetics to the adenosine A_3 receptor. Purinergic Signal. 15, 139-153.

Cao, J., Belousoff, M. J., Liang, Y. L., Johnson, R. M., Josephs, T. M., Fletcher, M. M., Christopoulos, A., Hay, D. L., Danev, R., Wootten, D., & Sexton, P. M. (2022). A structural basis for amylin receptor phenotype. Science *375*, eabm9609

Comeo, E., Kindon, N. D., Soave, M., Stoddart, L. A., Kilpatrick, L. E., Scammells, P. J., Hill, S. J., & Kellam, B. (2020). Subtype-Selective Fluorescent Ligands as Pharmacological Research Tools for the Human Adenosine A_{2A} Receptor. *Journal of Medicinal Chemistry*, *63*, 2656-2672.

Comeo, E., Trinh, P., Nguyen, A. T., Nowell, C. J., Kindon, N. D., Soave, M., Stoddart, L. A., White, J. M., Hill, S. J., Kellam, B., Halls, M. L., May, L. T., & Scammells, P. J. (2021). Development and Application of Subtype-Selective Fluorescent Antagonists for the Study of the Human Adenosine A_1 Receptor in Living Cells. Journal of Medicinal Chemistry, *64*, 6670-6695.

Comez D, Glenn J, Anbuhl SM, Heukers R, Smit MJ, Hill SJ, Kilpatrick LE. (2022) Fluorescently tagged nanobodies and NanoBRET to study ligand-binding and agonist-induced conformational changes of full-length EGFR expressed in living cells. Front Immunol. 13, 1006718.

Cooper SL, Soave M, Jörg M, Scammells PJ, Woolard J, Hill SJ. (2019) Probe dependence of allosteric enhancers on the binding affinity of adenosine A_1 -receptor agonists at rat and human A_1 -receptors measured using NanoBRET. Br J Pharmacol. 176, 864-878.

Copeland R. A. (2016). The drug-target residence time model: a 10-year retrospective. Nature Reviews Drug Discovery, *15*, 87–95.

Dale, N.C., Johnstone, E.K.M., White, C.W., and Pfleger, K.D.G. (2019). NanoBRET: The bright future of proximity-based assays. Front. Bioeng. Biotechnol. *7*: 1–13.

Dawson JP, Berger MB, Lin CC, Schlessinger J, Lemmon MA, Ferguson KM. (2005) Epidermal growth factor receptor dimerization and activation require ligand induced conformational changes in the dimer interface. Mol Cell Biol 25, 7734–42.

Díaz Ó, Martín V, Renault P, Romero D, Guillamon A, Giraldo J. (2023) Allosteric binding cooperativity in a kinetic context. Drug Discovery Today. 28,103441.

Dixon, A.S., Schwinn, M.K., Hall, M.P., Zimmerman, K., Otto, P., Lubben, T.H., et al. (2016). NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. ACS Chem. Biol. *11*, 400–408.

Draper-Joyce, C. J., Bhola, R., Wang, J., Bhattarai, A., Nguyen, A. T. N., Cowie-Kent, I., O'Sullivan, K., Chia, L. Y., Venugopal, H., Valant, C., Thal, D. M., Wootten, D., Panel, N., Carlsson, J., Christie, M. J., White, P. J., Scammells, P., May, L. T., Sexton, P. M., Danev,

R., … Christopoulos, A. (2021). Positive allosteric mechanisms of adenosine A_1receptor-mediated analgesia. Nature *597*, 571–576.

Endres NF, Das R, Smith AW, Arkhipov A, Kovacs E, Huang Y, Pelton JG, Shan Y, Shaw DE, Wemmer DE, Groves JT, Kuriyan J. (2013) Conformational coupling across the plasma membrane in activation of the EGF receptor. Cell, 152, 543-56.

Feuerstein TJ, Schlicker E. (2021) β_1-Blockers Enhance Inotropy of Endogenous Catecholamines in Chronic Heart Failure. Front Cardiovasc Med. 8, 639562.

Freed DM, Bessman NJ, Kiyatkin A, Salazar-Cavazos E, Byrne PO, Moore JO, et al. (2017) EGFR ligands differentially stabilize receptor dimers to specify signaling kinetics. Cell. 171:683–95.

Gao, Z. G., Van Muijlwijk-Koezen, J. E., Chen, A., Müller, C. E., Ijzerman, A. P., & Jacobson, K. A. (2001). Allosteric modulation of A(3) adenosine receptors by a series of 3-(2-pyridinyl)isoquinoline derivatives. Molecular Pharmacology, *60*, 1057–1063.

Gherbi, K., May, L. T., Baker, J. G., Briddon, S. J., & Hill, S. J. (2015). Negative cooperativity across β1-adrenoceptor homodimers provides insights into the nature of the secondary low-affinity CGP 12177 β1-adrenoceptor binding conformation. FASEB J *29*, 2859-2871.

Goulding J, Mistry SJ, Soave M, Woolard J, Briddon SJ, White CW, Kellam B, Hill SJ. (2021a) Subtype selective fluorescent ligands based on ICI 118,551 to study the human β2-adrenoceptor in CRISPR/Cas9 genome-edited HEK293T cells at low expression levels. Pharmacol Res Perspect., 9, e00779.

Goulding J, Kondrashov A, Mistry SJ, Melarangi T, Vo NTN, Hoang DM, White CW, Denning C, Briddon SJ, Hill SJ. (2021b) The use of fluorescence correlation spectroscopy to monitor cell surface β2-adrenoceptors at low expression levels in human embryonic stem cell-derived cardiomyocytes and fibroblasts. FASEB J. 35, e21398

Grätz, L., Müller, C., Pegoli, A., Schindler, L., Bernhardt, G., & Littmann, T. (2022). Insertion of Nanoluc into the extracellular loops as a complementary method to establish BRET-based binding assays for GPCRs. ACS Pharmacology Translational science, *5*, 1142–1155.

Grätz L, Sajkowska-Kozielewicz JJ, Wesslowski J, Kinsolving J, Bridge LJ, Petzold K, Davidson G, Schulte G, Kozielewicz P. (2023) NanoBiT- and NanoBiT/BRET-based assays allow the analysis of binding kinetics of Wnt-3a to endogenous Frizzled 7 in a colorectal cancer model. Br J Pharmacol. doi: 10.1111/bph.16090. Epub ahead of print.

Guo, D., Mulder-Krieger, T., IJzerman, A. P., & Heitman, L. H. (2012). Functional efficacy of adenosine A_2A receptor agonists is positively correlated to their receptor residence time. British Journal of Pharmacology, *166*, 1846–1859.

Hall, M. P., Unch, J., Binkowski, B. F., Valley, M. P., Butler, B. L., Wood, M. G., Otto, P., Zimmerman, K., Vidugiris, G., Machleidt, T., Robers, M. B., Benink, H. A., Eggers, C. T., Slater, M. R., Meisenheimer, P. L., Klaubert, D. H., Fan, F., Encell, L. P., & Wood, K. V. (2012). Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. ACS Chemical Biology, *7*, 1848–1857.

Hay, D. L., Chen, S., Lutz, T. A., Parkes, D. G., & Roth, J. D. (2015). Amylin: Pharmacology, Physiology, and Clinical Potential. Pharmacological Reviews, *67*, 564–600.

Hounsou C, Margathe JF, Oueslati N, Belhocine A, Dupuis E, Thomas C, Mann A, Ilien B, Rognan D, Trinquet E, Hibert M, Pin JP, Bonnet D, Durroux T. (2015) Time-resolved FRET binding assay to investigate hetero-oligomer binding properties: proof of concept with dopamine D1/D3 heterodimer. ACS Chem Biol. 10, 466-74.

Hounsou C, Baehr C, Gasparik V, Alili D, Belhocine A, Rodriguez T, Dupuis E, Roux T, Mann A, Heissler D, Pin JP, Durroux T, Bonnet D, Hibert M. (2018) From the Promiscuous Asenapine to Potent Fluorescent Ligands Acting at a Series of Aminergic G-Protein-Coupled Receptors. J Med Chem. 61, 174-188.

IJzerman AP, Guo D. (2019) Drug-target association kinetics in drug discovery. Trends Biochem Sci. 44, 861-871

Kaumann, A. J., and Molenaar, P. (2008) The low-affinity site of the b1-adrenoceptor and its relevance to cardiovascular pharmacology. Pharmacol. Ther. 118, 303–336

Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K. (2003) A general method for the covalent labeling of fusion proteins with small molecules in vivo. Nat Biotechnol. 21, 86-9.

Kilpatrick, L. E., Friedman-Ohana, R., Alcobia, D. C., Riching, K., Peach, C. J., Wheal, A. J., Briddon, S. J., Robers, M. B., Zimmerman, K., Machleidt, T., Wood, K. V., Woolard, J., & Hill, S. J. (2017). Real-time analysis of the binding of fluorescent $VEGF_{165}$a to VEGFR2 in living cells: Effect of receptor tyrosine kinase inhibitors and fate of internalized agonist-receptor complexes. Biochemical Pharmacology, *136*, 62–75.

Kilpatrick LE, Alcobia DC, White CW, Peach CJ, Glenn JR, Zimmerman K, Kondrashov A, Pfleger KDG, Ohana RF, Robers MB, Wood KV, Sloan EK, Woolard J, Hill SJ. (2019) Complex Formation between VEGFR2 and the β_2-Adrenoceptor. Cell Chem Biol. 26, 830-841.

Kok ZY, Stoddart LA, Mistry SJ, Mocking TAM, Vischer HF, Leurs R, Hill SJ, Mistry SN, Kellam B. (2022) Optimization of Peptide Linker-Based Fluorescent Ligands for the Histamine H_1Receptor. J Med Chem. 65, 8258-8288.

Kostenis, E., Botero Cid, H. M., Holzgrabe, Y., & Mohr, K. (1996). Evidence for a multiple binding mode of bispyridinium-type allosteric modulators of muscarinic receptors. European Journal of Pharmacology, *314*, 385–392.

Lay, C.S., Bridges, A., Goulding, J., Briddon, S.J., Soloviev, Z., Craggs, P.D., et al. (2022). Probing the binding of interleukin-23 to individual receptor components and the IL-23 heteromeric receptor complex in living cells using NanoBRET. Cell Chem. Biol. *29*: 19–29.

Lay CS, Isidro-Llobet A, Kilpatrick LE, Craggs PD, Hill SJ. (2023a) Characterisation of IL-23 receptor antagonists and disease relevant mutants using fluorescent probes. Nat Commun. 14, 2882.

Lay CS, Kilpatrick LE, Craggs PD, Hill SJ. (2023b) Use of NanoBiT and NanoBRET to characterise interleukin-23 receptor dimer formation in living cells. Br J Pharmacol. 180, 1444-1459.

Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohana RF, Urh M, Simpson D, Mendez J, Zimmerman K, Otto P, Vidugiris G, Zhu J, Darzins A, Klaubert DH, Bulleit RF, Wood KV. (2008) HaloTag: a novel protein labeling technology for cell imaging and protein analysis. ACS Chem Biol. 3, 373-82.

May LT, Self TJ, Briddon SJ, Hill SJ. (2010) The effect of allosteric modulators on the kinetics of agonist-G protein-coupled receptor interactions in single living cells. Mol Pharmacol. 78:511-23.

May LT, Bridge LJ, Stoddart LA, Briddon SJ, Hill SJ. (2011) Allosteric interactions across native adenosine-A3 receptor homodimers: quantification using single-cell ligand-binding kinetics. FASEB J 25, 3465-76.

Molderings, G. J., Menzel, S., Kathmann, M., Schlicker, E., & Göthert, M. (2000). Dual interaction of agmatine with the rat alpha(2D)-adrenoceptor: competitive antagonism and allosteric activation. British Journal of Pharmacology, *130*, 1706–1712

Parham, C. *et al.* (2022) A Receptor for the Heterodimeric Cytokine IL-23 Is Composed of IL-12Rβ1 and a Novel Cytokine Receptor Subunit, IL-23R. J. Immunol. 168, 5699–5708.

Peach CJ, Kilpatrick LE, Friedman-Ohana R, Zimmerman K, Robers MB, Wood KV, Woolard J, Hill SJ. (2018a) Real-Time Ligand Binding of Fluorescent VEGF-A Isoforms that Discriminate between VEGFR2 and NRP1 in Living Cells. Cell Chem Biol. 25, 1208-1218.

Peach CJ, Mignone VW, Arruda MA, Alcobia DC, Hill SJ, Kilpatrick LE, Woolard J. (2018b) Molecular Pharmacology of VEGF-A Isoforms: Binding and Signalling at VEGFR2. Int J Mol Sci. 19, 1264.

Peach CJ, Kilpatrick LE, Woolard J, Hill SJ. (2021) Use of NanoBiT and NanoBRET to monitor fluorescent VEGF-A binding kinetics to VEGFR2/NRP1 heteromeric complexes in living cells. Br J Pharmacol. 178, 2393-2411.

Rose RH, Briddon SJ, Hill SJ. (2012) A novel fluorescent histamine H(1) receptor antagonist demonstrates the advantage of using fluorescence correlation spectroscopy to study the binding of lipophilic ligands. Br J Pharmacol. 165, 1789-1800.

Schuetz, D. A., de Witte, W. E. A., Wong, Y. C., Knasmueller, B., Richter, L., Kokh, D. B., Sadiq, S. K., Bosma, R., Nederpelt, I., Heitman, L. H., Segala, E., Amaral, M., Guo, D., Andres, D., Georgi, V., Stoddart, L. A., Hill, S., Cooke, R. M., De Graaf, C., Leurs, R., … Ecker, G. F. (2017). Kinetics for Drug Discovery: an industry-driven effort to target drug residence time. Drug Discovery Today, *22*, 896–911

Schiele, F., Ayaz, P., & Fernández-Montalván, A. (2015). A universal homogeneous assay for high-throughput determination of binding kinetics. Analytical Biochemistry, *468*, 42–49.

Suchankova, A., Harris, M., & Ladds, G. (2021). Measuring the rapid kinetics of receptor-ligand interactions in live cells using NanoBRET. Methods in Cell Biology, *166*, 1–14.

Sivanesan, D., Beauchamp, C., Quinou, C., Lee, J., Lesage, S., Chemtob, S., et al. (2016). IL23R (Interleukin 23 Receptor) variants protective against inflammatory bowel diseases (IBD) display loss of function due to impaired protein stability and intracellular trafficking. J. Biol. Chem. *291*, 8673–8685.

Soave M, Stoddart LA, White CW, Kilpatrick LE, Goulding J, Briddon SJ, Hill SJ. (2021) Detection of genome-edited and endogenously expressed G protein-coupled receptors. FEBS J. 288, 2585-2601.

Stoddart LA, Johnstone EKM, Wheal AJ, Goulding J, Robers MB, Machleidt T, Wood KV, Hill SJ, Pfleger KDG.(2015) Application of BRET to monitor ligand binding to GPCRs. Nat Methods. 12, 661-663.

Stoddart LA, White CW, Nguyen K, Hill SJ, Pfleger KD. (2016) Fluorescence- and bioluminescence-based approaches to study GPCR ligand binding. Br J Pharmacol. 173, 3028-37.

Stoddart LA, Kilpatrick LE, Hill SJ. (2018a) NanoBRET Approaches to Study Ligand Binding to GPCRs and RTKs. Trends Pharmacol Sci. 39, 136-147.

Stoddart LA, Vernall AJ, Bouzo-Lorenzo M, Bosma R, Kooistra AJ, de Graaf C, Vischer HF, Leurs R, Briddon SJ, Kellam B, Hill SJ. (2018b) Development of novel fluorescent histamine H_1-receptor antagonists to study ligand-binding kinetics in living cells. Sci Rep. 8, 1572.

Sykes DA, Charlton SJ. (2018) Single step determination of unlabeled compound kinetics using a competition association binding method employing time-resolved FRET. Methods Mol Biol. 1824, 177-194.

Sykes DA, Moore H, Stott L, Holliday N, Javitch JA, Lane JR, Charlton SJ. (2017) Extrapyramidal side effects of antipsychotics are linked to their association kinetics at dopamine D_2 receptors. Nat Commun. 8, 763.

Sykes DA, Stoddart LA, Kilpatrick LE, Hill SJ. (2019) Binding kinetics of ligands acting at GPCRs. Mol Cell Endocrinol. 485, 9-19.

Toy, L., Huber, M. E., Schmidt, M. F., Weikert, D., & Schiedel, M. (2022). Fluorescent Ligands Targeting the Intracellular Allosteric Binding Site of the Chemokine Receptor CCR2. ACS Chemical Biology, *17*, 2142–2152.

Van den Bor J, Bergkamp ND, Anbuhl SM, Dekker F, Comez D, Perez Almeria CV, Bosma R, White CW, Kilpatrick LE, Hill SJ, Siderious M, Smit MJ, Heuker R (2023) NanoB2 to monitor interactions of ligands with membrane proteins by conbining nanobodies and NanoBRET. Cell Reports Methods 3, 100422.

van der Westhuizen, E. T., Valant, C., Sexton, P. M., & Christopoulos, A. (2015). Endogenous allosteric modulators of G protein-coupled receptors. The Journal of Pharmacology and Experimental Therapeutics, *353*, 246–260.

Vernall AJ, Hill SJ, Kellam B. (2014) The evolving small-molecule fluorescent-conjugate toolbox for Class A GPCRs. Br J Pharmacol. 171, 1073-84.

Wesslowski J, Kozielewicz P, Wang X, Cui H, Schihada H, Kranz D, Karuna M P, Levkin P, Gross JC, Boutros M, Schulte G, Davidson G. (2020) eGFP-tagged Wnt-3a enables functional analysis of Wnt trafficking and signaling and kinetic assessment of Wnt binding to full-length Frizzled. J Biol Chem. 295, 8759-8774.

White CW, Caspar B, Vanyai HK, Pfleger KDG, Hill SJ. (2020) CRISPR-Mediated Protein Tagging with Nanoluciferase to Investigate Native Chemokine Receptor Function and Conformational Changes. Cell Chem Biol. 27, 499-510.

White CW, Vanyai HK, See HB, Johnstone EKM, Pfleger KDG. (2019) Using nanoBRET and CRISPR/Cas9 to monitor proximity to a genome-edited protein in real-time. Sci Rep. 7, 3187.

White C, Rottschäfer V, Bridge LJ. (2022) Insights into the dynamics of ligand-induced dimerisation via mathematical modelling and analysis. J Theor Biol. 538:110996.

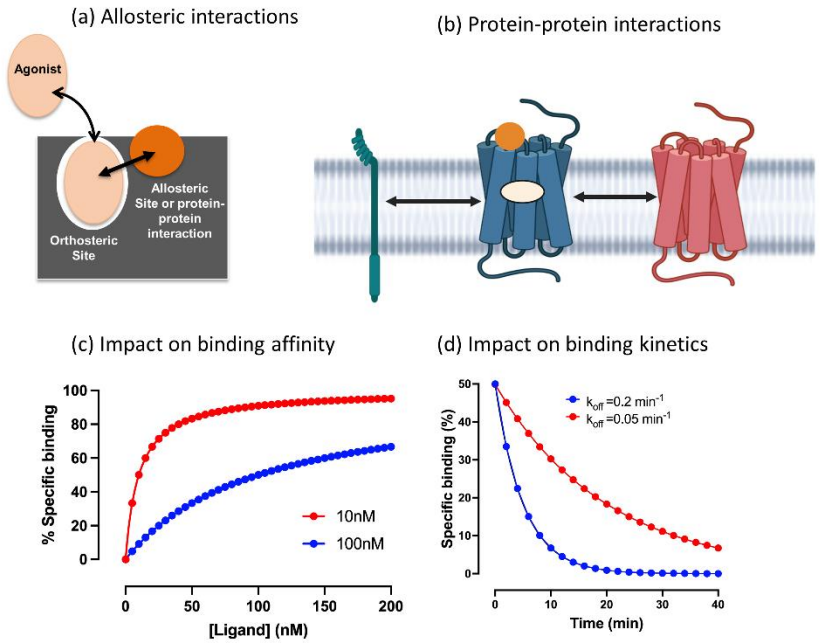


Figure 1. Allosteric influence of allosteric modulators and protein-protein interactions on the binding of a ligand to the orthosteric binding site of a GPCR. (a,b) Schematic showing the potential for (a) allosteric modulators and (b) neighbouring proteins (e.g. single membrane spanning proteins or other GPCRs) to exert allosteric influences on the binding of an orthosteric ligand. (b) Simulated data illustrating the impact of a positive allosteric modulator (red circles) on the specific binding of an orthosteric ligand (blue circles) that changes the K_D of the orthosteric ligand from 100nM to 10nM. (c) The impact of a positive allosteric modulator (red circles) that decreases the k_{off} rate constant of an orthosteric ligand (blue circles) from 0.2 min^{-1} to 0,05 min^{-1}.

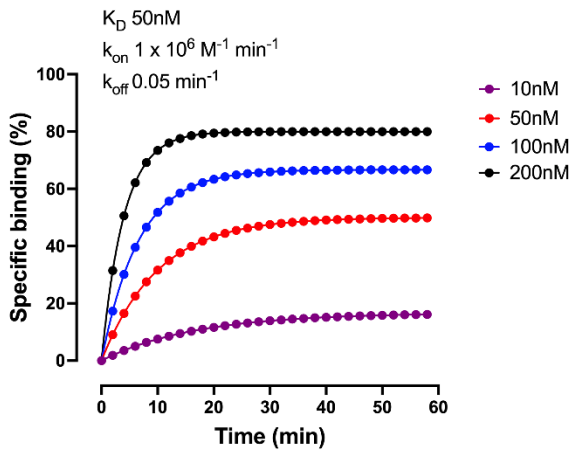
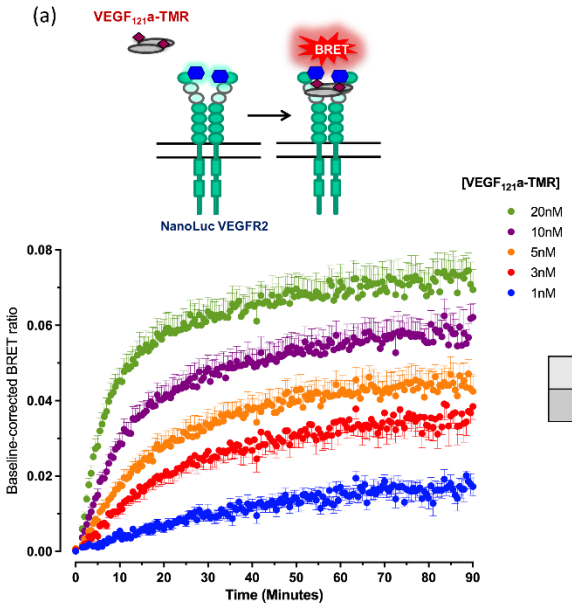
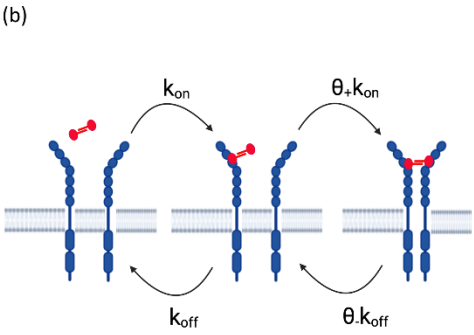


Figure 2. Simulated data for the specific binding of increasing concentrations of an orthosteric ligand to a target receptor. The simulations were run with a K_D of 50 nM, k_{on} of 1 x 10^6 M^{-1}.min^{-1} and k_{off} of 0.05 min^{-1}.





k_{on} ($min^{-1}M^{-1}$)	k_{off} (min^{-1})	$\theta+$($min^{-1}M^{-1}$)	$\theta-$ (min^{-1})
$2.23x10^6$	0.059	2.61×10^4	1.63×10^3

Figure 3. Binding kinetics of increasing concentrations of $VEGF_{121}$a-TMR in membrane preparations quantified using NanoBRET. (a) Binding of $VEGF_{121}$a-TMR to membranes prepared from NanoLuc-VEGFR2-overexpressing HEK293T cells. Membranes were pretreated with furimazine to equilibrate for 5 min before addition of five concentrations of $VEGF_{121}$a-TMR (1–20 nM). Data taken from Peach et al (2019). BRET ratios are expressed as mean ± SEM from six independent experiments with duplicate wells. Analysis assuming a simple mass-action equilibrium gave estimated k_{on} and k_{off} values of 5.13×10^6 $M^{-1}.min^{-1}$ and 0.019 min^{-1} respectively (Peach et al., 2019). (b) Analysis of the data set from Peach et al (2019) based on the ligand-induced homodimerization model of White et al., (2022). The first phase of binding to one VEGFR monomer can be described by simple mass action kinetics involving k_{on} and k_{off}. This is then followed by a second phase involving ligand-induced dimerization with the cooperativity rate constants (θ_+ and θ_-) describing the binding to the second VEGFR2 protomer. The table shows the rate constants determined from this analysis by White et al (2022).

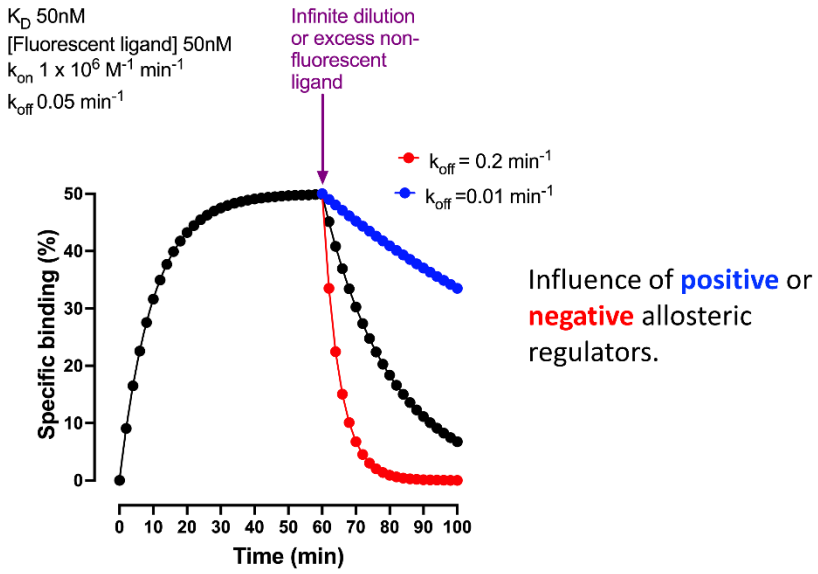
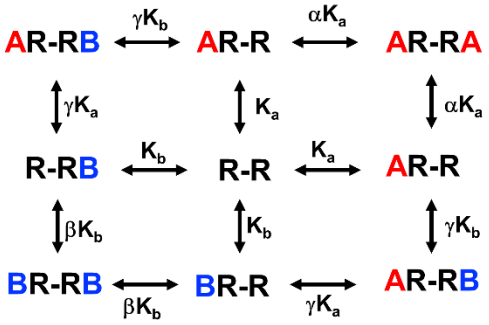


Figure 4. Simulated data for the effect of positive and negative allosteric modulators on the dissociation kinetics of a fluorescent orthosteric ligand. In this simulation, the fluorescent ligand was allowed to reach equilibrium before it was washed away. The black circles show the association and dissociation phases of binding of the fluorescent orthosteric ligand with a k_{on} of 1 x 10^6 $M^{-1}.min^{-1}$ and a k_{off} of 0.05 min^{-1}. The red and blue circles show the impact on the dissociation phase of binding of a high concentration of a negative (red) or positive (blue) allosteric modulator is added at the start of the infinite dilution phase.



Agonist/Antagonist	pK_D	α	β	γ
ABA-X-BY630	7.9	<0.01		
NECA	7.0		0.18	0.13
XAC	7.6		0.02	0.07

Figure 5. A two-drug model for constitutive GPCR homodimerization. In this model it is assumed that the receptor can exist as a dimeric species R-R. Ligand A (red) and ligand B (blue) can bind to the first and/or second protomer of the homodimer. Binding of ligand A and ligand B is defined by their affinity for the free GPCR dimer (R-R) with K_a and K_b being their dissociation constants respectively. The cooperativity factors α, β and γ then describe the impact of a ligand (A or B) binding to the second protomer of an already occupied homodimer. The table shows the fitted binding constants for this model for the binding of the fluorescent adenosine ligand ABA-X-BY630, the agonist NECA and the antagonist XAC to the human adenosine A_3-receptor obtained from the data of May et al., (2011).

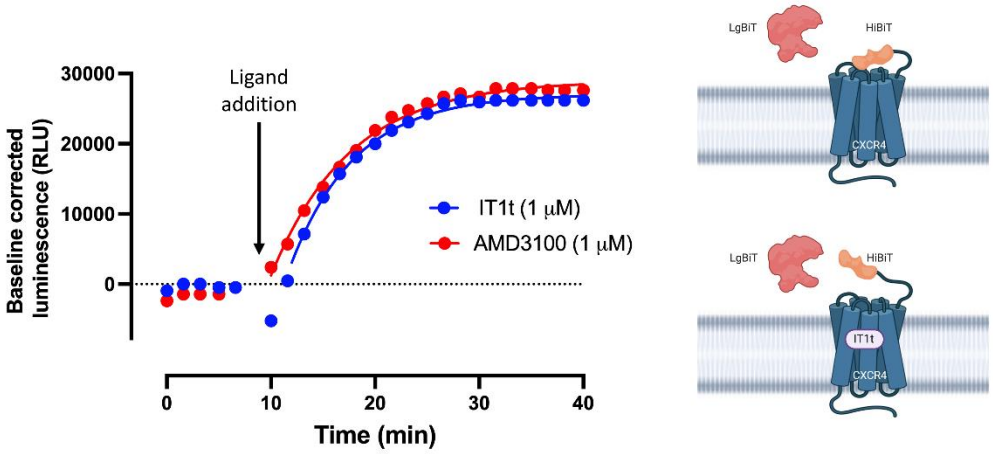


Figure 6. Time course of conformational changes induced by IT1t (blue circles) and AMD3100 (red circles) for HiBiT-tagged CXCR4 receptors in membrane preparations. Data taken from White et al., 2020. Luminescence was measured using membrane preparations from HEK 293 cells exogenously expressing HiBiT-CACR4 and preincubated with 10nM purified LgBiT.

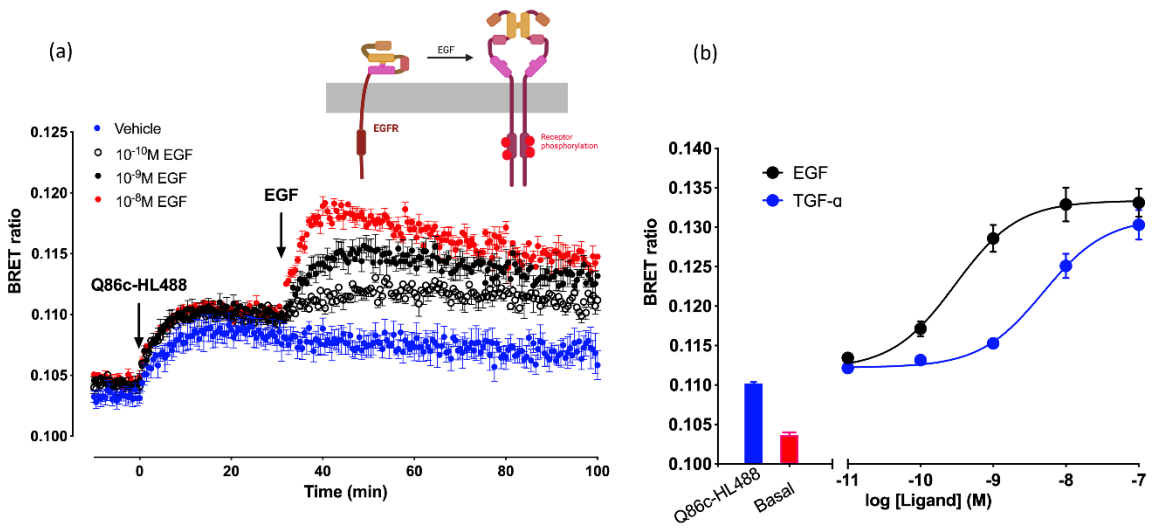


Figure 7. Kinetics of EGF-induced changes in the conformation of NanoLuc-tagged EGFR bound to the fluorescent nanobody Q86c-HL488. (a) NanoBRET was monitored between NanoLuc-tagged EGFR and the fluorescent nanobody (25nM) added at time zero. Once equilibrium binding of Q86c-HL488 had been established, increasing concentrations of EGF or vehicle were added. Data are taken from Comez et al., 2022. (b) Concentration response data for EGF and TGF-α stimulation of the NanoBRET signal obtained with Q86c-HL488 and NanoLuc-EGFR after 30 min stimulation. Data taken from Comez et al., 2022.