Published in final edited form as:

Nat Protoc. 2011 March ; 6(3): 365–387. doi:10.1038/nprot.2011.305.

Analysis of protein-ligand interactions by fluorescence polarization

Ana M. Rossi and Colin W. Taylor

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1PD, UK.

Abstract

Quantification of the associations between biomolecules is required both to predict and understand the interactions that underpin all biological activity. Fluorescence polarization (FP) provides a non-disruptive means of measuring the association of a fluorescent ligand with a larger molecule. We describe an FP assay in which binding of fluorescein-labelled inositol 1,4,5-trisphosphate (IP₃) to N-terminal fragments of IP₃ receptors can be characterised at different temperatures and in competition with other ligands. The assay allows the standard Gibbs free energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) changes of ligand binding to be determined. The method is applicable to any purified ligand-binding site for which an appropriate fluorescent ligand is available. FP can be used to measure low-affinity interactions in real-time without use of radioactive materials, it is non-destructive, and with appropriate care it can resolve ΔH° and ΔS° . The first part of the protocol, protein preparation, may take several weeks, while the FP measurements, once they have been optimised, would normally take 1–6 h.

Keywords

Enthalpy; Entropy; Fluorescein; Fluorescence polarization; Fluorescent ligand; Gibbs free energy; IP₃ receptor; Ligand binding

INTRODUCTION

Life, from the simplest bacterium to the most complex multicellular animal, depends on specific interactions between molecules. Replication and decoding of genetic material, for example, require selective pairing of nucleotide sequences guided by specific interactions with proteins. Assembly of the macromolecular complexes that establish cellular architecture, and the biochemical pathways that sustain cells are each determined by selective interactions between proteins, nucleic acids and lipids. Every biochemical step within a cell, from enzyme catalysis to ion transport and receptor activation, is initiated by specific interactions between a macromolecule and a *ligand*. The latter (from *ligo*, to bind) has come to mean rather different things to chemists and biologists. Here we define a *ligand* as any species (ion, molecule, etc) that binds selectively, stoichiometrically and reversibly to a larger molecule, usually a macromolecule like DNA or protein. The interactions between biomolecules is determined by their concentrations (and therefore their subcellular distributions) and by the strength of the interactions between them. Quantitative analysis of

Correspondence should be addressed to C.W.T. (cwt1000@cam.ac.uk)..

AUTHOR CONTRIBUTION Both authors wrote the paper and supervised the original project.

COMPETING INTEREST STATEMENT The authors declare that they have no competing financial interests.

the interactions between biomolecules is therefore central to understanding every aspect of cellular behaviour.

Quantification of protein-ligand interactions

Simple rate equations describe the interactions between any two molecules (D and R):

The rates of the two reactions are given by the products of the concentrations of the reactants (denoted [D], etc; abbreviations and definitions are listed in Table 1) and the appropriate rate constant (k_{+1} or k_{-1}):

$$\begin{aligned} \text{Forward rate} &= k_{+1} [D] [R] \\ \text{Backward rate} &= k_{-1} [DR] \end{aligned}$$

At equilibrium, both reactions occur at the same rate and so:

$$\begin{aligned} D+R &\leftrightarrow DR \\ k_{+1} [D] [R] &= k_{-1} [DR] \end{aligned}$$

hence:

$$K_A = \frac{k_{+1}}{k_{-1}} = \frac{[DR]}{[D][R]} \quad (1)$$

Whether K_A (equilibrium association constant) or the equilibrium dissociation constant ($K_D = 1/K_A = [D][R]/[DR]$) is chosen to define the strength (or *affinity*) of the interaction is a matter of preference. Henceforth, we use K_D . In these equations, concentrations refer, of course, to the *free* concentrations of D and R, but as D and R associate to form DR, their free concentrations must decrease. If the concentration of one reactant massively exceeds that of the other (e.g. $D_T \gg R_T$), their association will significantly affect the free concentration of only the less concentrated reactant (R in this case). If, however, the total concentrations of the partners are both similar and sufficient to allow them to bind ($D_T \approx R_T > K_D$), their association will significantly reduce the free concentration of both. In cells, both situations occur. However, in most experimental analyses of ligand binding, conditions are chosen to ensure that the concentration of one reactant substantially exceeds that of the other. For conventional analyses of radioligand binding to receptors, for example, the concentration of radioligand (D_T) used considerably exceeds the total concentration of receptors (R_T), so that no matter how many receptors bind ligand, the free concentration of ligand is not significantly changed^{1,2}. In this situation, where $D_T \approx [D]$ and $[R] = R_T - [DR]$:

$$K_D = \frac{[D][R]}{[DR]}$$

hence:

$$K_D = \frac{[D](R_T - [DR])}{[DR]}$$

and:

$$\frac{[DR]}{R_T} = \frac{\frac{[D]}{K_D}}{1 + \frac{[D]}{K_D}} \quad (2)$$

$[DR]/R_T$ is the *fractional occupancy* (often abbreviated, α). We now have a means of determining K_D by measuring how α varies as a function of $[D]$ (Fig. 1a). Note that when α is 0.5 (i.e. 50 % of R have bound D), $[D] = K_D$. Note too how $[D]$ is invariably expressed relative to its affinity for R (i.e. $[D]/K_D$).

We can measure the K_D of a bimolecular interaction by measuring the two rate constants (k_{+1} and k_{-1}) or more easily by measuring the saturable relationship between $[D]$ and $[DR]$ at *equilibrium* (Fig. 1a). The latter requires that we identify, without appreciably perturbing the equilibrium, the *small* fraction of D that has bound to R (a *small* fraction because we usually ensure that $D_T \gg R_T$).

Not all binding reactions are so simple. Binding sites may interact (cooperativity), or the initial complex of ligand and receptor may undergo conformational changes or interactions with additional partners that influence the strength of the binding event^{3,4}. These are important issues, but they need not deflect us from the focus of this protocol, which is to describe the practicalities of measuring the K_D for a simple ligand-receptor interaction.

Thermodynamic analysis of protein-ligand interactions

The K_D provides a measure of the strength of the interaction between the two partners and allows us to predict how their association will change as their concentrations vary (Fig. 1a). We can also interpret the K_D from a thermodynamic perspective and so, with appropriate experiments, determine the contributions of standard enthalpy (ΔH°) and entropy (ΔS°) changes to the binding event. The Second Law of Thermodynamics, and thereby the relationships between the standard Gibbs free energy change (ΔG°), ΔH° , ΔS° and K_D , provides the route to these analyses⁵:

$$\Delta G^\circ = RT \ln K_D \quad (3)$$

$$\Delta G^\circ = \Delta H^\circ - T \Delta S^\circ \quad (4)$$

where: R is the universal gas constant, and T is the absolute temperature.

The Second Law, which dictates that reactions proceed spontaneously in the direction of decreasing free energy ($\Delta G < 0$) (Fig. 1b), makes the relationship between ΔG° and K_D intuitively clear^{5,6}. When there is no difference in free energy between reactants and products, there is no driving force for their association or dissociation ($\Delta G^\circ = 0$ and so $K_D = 1$). When the products have more free energy than the reactants, the driving force is towards dissociation ($\Delta G^\circ > 0$, $K_D > 1$), but when the reactants have more free energy than the products, the driving force is towards association ($\Delta G^\circ < 0$, $K_D < 1$) (Fig. 1b).

It is not easy to relate the two components of ΔG° , ΔH° and $T \Delta S^\circ$, specifically and directly to binding mechanisms⁷⁻⁹. Broadly, ΔH° reflects energy changes associated with making

($-\Delta H^\circ$) and breaking ($+\Delta H^\circ$) bonds, while ΔS° reflects changes associated with increasing ($+\Delta S^\circ$) or decreasing ($-\Delta S^\circ$) the number of microscopic configurations available to the system. ΔS° therefore reflects the extent to which binding changes such features as the conformational flexibility or mobility of the reactants, products and solvent. Empirically, it has been reported that for some ligand-receptor interactions, the relative contributions of ΔH° and ΔS° to binding may distinguish those ligands that activate a receptor (*agonists*) from those that bind without activating (*competitive antagonists*)^{7,10}. Clearly measurements of K_D (and so ΔG°) are not alone sufficient to resolve the contributions from ΔH° and ΔS° . That requires either measurements at different temperatures or direct measurement of ΔH° (Table 2).

Methods to measure ligand binding

Among the factors to consider when deciding on the most suitable method for quantitative analysis of a protein-ligand interaction are the following:

1. Does the analysis need to be performed in an intact cell or can it be performed in a disrupted system?
2. Is the interaction strong enough (more specifically is k_{-1} slow enough) to allow separation of free and bound ligand without appreciably perturbing the equilibrium?
3. Does the interaction require use of an unmodified ligand or are modified ligands (e.g., radioactive, fluorescent, etc) available and useful? If the method requires purified protein, is it functional and available in sufficient quantity?
4. Is measurement of the K_D sufficient or is there also need to determine either rate constants (k_{+1} and k_{-1}) or ΔH° and ΔS° ?

The means whereby bound ligand is detected divides the methods into two broad categories (Table 2). The first requires that bound and free ligand are separated (usually by filtration or centrifugation), while the second detects bound ligand without physically separating it from the free ligand. The latter can measure even low-affinity interactions because there is no separation step during which fast ligand dissociation (large k_{-1}) might perturb the equilibrium. These methods quantify bound ligand by directly measuring ΔH (isothermal titration calorimetry, ITC), by detecting the increase in size of the protein-ligand complex (fluorescence polarization, FP; surface plasmon resonance, SPR; fluorescence correlation spectroscopy, FCS), by bringing the ligand into contact with a sensor when it binds to an immobilised protein (scintillation proximity assay, SPA; total internal reflection fluorescence microscopy, TIRFM), or by detecting a change in fluorescence lifetime (fluorescence lifetime imaging microscopy, FLIM) or fluorescence spectrum (fluorescence resonance energy transfer, FRET) when a fluorescent ligand associates with a protein, which must itself be fluorescent for FRET. The advantages and limitations of these different methods are compared in Table 2.

Protein-ligand interactions analysed by fluorescence polarization

When a rigid and entirely immobile fluorophore is excited (typically in 10^{-15} s) by plane-polarized light, it emits light in orientations that depend upon the angle (ζ) between the

orientations of the absorbing and emitting dipoles¹¹: $A = \frac{3 \cos^2 \zeta - 1}{5}$. If the dipoles are parallel ($\zeta = 0^\circ$), for example, then $A = 0.4$ (i.e. 60 % of emitted light would be detected in the same plane as the exciting light, and 20 % in each of the other two planes) (Fig. 2a). If there were no rotation of such a molecule during the lifetime of the excited state of the fluorophore (typically ns), then the maximal value of A would be 0.4. But if the molecule

rotates (<ns timescales for small molecules), it will no longer be aligned, and when the fluorophore emits its light less than 60 % will be aligned with the source (Fig. 2a). The speed at which the fluorophore tumbles (defined by its rotational relaxation time, ρ) relative to the lifetime of its excited state (the interval between absorbing and emitting a photon) will determine whether rotation of a molecule can be detected¹². Fluorescence polarization (FP) measures the light emitted from a fluorescent ligand in two planes (horizontal and vertical) after excitation with plane-polarized light in one of these planes (Fig. 2b)¹¹. The intensities of the light detected in the parallel ($I_{||}$) and perpendicular ($I_{\perp}$) planes with respect to the excitation light are then related to the anisotropy (A):

$$A = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \quad (5)$$

A for an immobile fluorescent molecule with parallel transition dipoles is therefore¹¹:

$$A = \frac{60 - 20}{60 + 40} = 0.4$$

Others use polarization ($P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$) to define the relationship between the intensities of the detected light, but A is preferable because it is more arithmetically convenient. Although P is often reported as mP ($P/1000$), both A and P are dimensionless quantities, the use of milliunits should therefore be avoided. If a molecule tumbles so that it becomes randomly re-oriented during its fluorescent lifetime, then A becomes 0 (because $I_{||} = I_{\perp}$). A for a mixture of fluorescent molecules is given by¹¹:

$$A = \sum_{i=1}^n f_i A_i$$

where each fluorescent species has anisotropy A_i , and a fractional fluorescence intensity f_i . In practise, therefore, A decreases linearly from a maximum of 0.4 to a minimal value of 0 as the fraction of re-oriented molecules increases. For a spherical molecule, the rotational relaxation time (ρ) is described by the Stokes' equation:

$$\rho = \frac{3\eta V}{RT}$$

where ρ , the rotation relaxation time (time taken for the molecule to rotate through 68.5°); V , molecular volume; η , viscosity; R and T have their usual meanings. With other conditions constant, the speed of tumbling is inversely related to molecular volume. A large molecule is, therefore, likely to retain the same orientation during the interval (~4 ns for fluorescein)¹³ between absorbing and emitting a photon, while a smaller one is more likely to re-orient. Binding of a small fluorescent ligand (D^*) to a large protein (R) therefore increases A . The measured A (A_M) is then the sum of that due to free D^* (A_{D^*}) and that due to D^* bound to R (A_{D^*R}):

$$A_M = F_F A_{D^*} + F_B A_{D^*R} \quad (6)$$

where F_F and F_B are the fractions of free and bound D^* , respectively ($F_F + F_B = 1$). This then provides the basis for using FP as a non-disruptive assay of ligand binding (Fig. 2c).

Although the method is applicable to analyses of binding of any small fluorescent ligand to any larger molecule¹⁴, we focus on ligand binding (D) to a receptor (R). There are, however, three important differences between FP analyses and conventional assays of radioligand binding that influence both experimental design and analysis. First, FP requires a fluorescent ligand (D^*). Second, although FP does not require separation of bound and free D^* , it does require that we distinguish them by their fluorescence. Third, FP analyses require that a substantial amount of D^* is bound to R to provide a measurable change in A . We cannot, therefore assume, as we do with radioligand binding assays, that $D^*_T \approx [D^*]$. The effects of these differences on the design of FP experiments are described in Box 1.

Applications of FP assays

FP binding assays can, in principle, be used quantitatively to analyse binding of any small soluble fluorescent molecule (and any soluble ligand that competes with it) to a larger soluble protein. The suitability of FP assays to high-throughput screening using relatively inexpensive equipment has led to their use in screening programmes in drug discovery and as 'off the shelf' assay kits for a variety of biomolecules. The former include development of inhibitors of interactions between proteins and either small molecules¹⁵⁻¹⁹ or other proteins²⁰. Use of FP to detect antibody-antigen interactions²¹ has provided many opportunities to adapt FP assays for bioassay of enzyme activity²², bioactive proteins²³ and intracellular messengers²⁴ and metabolites²⁵. Our FP assay was developed to allow analysis of interactions between N-terminal fragments of the IP₃R and small-molecule agonists. It can also be used also to assay levels of endogenous IP₃ extracted from biological samples²⁴.

Advantages and limitations of FP assays

FP assays do not require separation of bound and free ligand. This allows ligand binding to be quantified without perturbing the equilibrium, making it suitable for measurement of low-affinity interactions (more specifically, interactions with fast dissociation rates). Although the initial optimization of FP assays is likely to be time-consuming, thereafter they are easily automated. They can, therefore, provide high-throughput, economical, rapid and convenient screens for large numbers of unlabelled ligands. FP assays are non-destructive, thereby allowing repetitive measurements of the same sample under different conditions (e.g., different temperatures). Such analyses, which would usually be impracticable with radioligand binding assays, allow the contributions from ΔH° and $T\Delta S^\circ$ to ΔG° to be resolved. Other methods, like ITC (Table 2), can provide more direct, and perhaps more reliable, access to these thermodynamic characteristics of protein-ligand interactions, but they require large amounts of pure protein and more costly equipment. FP assays avoid routine use of radioligands, with consequent financial, health and environmental benefits. All FP assays require a fluorescent ligand. This may be available commercially or it may require development of procedures for conjugation of the ligand to a reactive fluorophore (many of these are available from Invitrogen, Sigma and other suppliers) and subsequent purification of the conjugate. It may even be necessary (as it was with the FITC-IP₃ used in our assay) to synthesise a form of the ligand suitable for conjugation¹⁸. Whereas radiolabelling of a ligand (particular with ³H) is unlikely to affect its interaction with a target protein, fluorophores are likely to affect the interaction. In the worse case, it may be impracticable to obtain a fluorescent ligand that interacts effectively with a target protein; FP would then be impossible. Every FP assay requires accurate quantification of the concentration of functional binding sites. This is manageable when relatively few proteins are to be used to screen a battery of ligands, but it may become impracticable if the aim is to screen a large number of proteins (e.g. mutants). FP assays are more demanding than radioligand binding in the amount of protein they require, but less so than other techniques, like SPR or ITC. Specialised, though relatively inexpensive, equipment is required for FP and available equipment can often be used also for other optical assays. Finally, FP assays

are applicable only to soluble partners and only for fluorescent ligands that are considerably smaller than the protein to which they bind. Table 2 compares the advantages and limitations of the most common methods used to characterise protein-ligand interactions.

IP₃ receptors

Our use of FP to measure ligand binding has focussed on inositol 1,4,5-trisphosphate receptors (IP₃R). Before exploring the methods, we therefore provide some essential background information on these intracellular Ca²⁺ channels²⁶. IP₃R are expressed in the membranes of the endoplasmic reticulum of most, if not all, animal cells. They mediate the initial release of Ca²⁺ from intracellular stores evoked by the many cell-surface receptors that stimulate IP₃ formation. Because IP₃R are also regulated by the Ca²⁺ they release, they can also initiate regenerative Ca²⁺ signals, allowing the initial openings of a few IP₃R to grow into Ca²⁺ waves that may invade the entire cell^{27,28}. The important point is that activation of all IP₃R is initiated by IP₃ binding; this then promotes Ca²⁺ binding, which then leads to opening of the Ca²⁺-permeable pore. All IP₃R are large tetrameric proteins, each subunit of which includes a very large cytoplasmic region, six transmembrane domains towards the C-terminus that contribute to the pore, and a short C-terminal tail (Fig. 3a). The IP₃-binding core (IBC, residues 224-604 of IP₃R) of each subunit is entirely responsible for binding IP₃. A high-resolution structure of the IBC shows IP₃ cradled within a clam-like structure lined with many basic residues (Fig. 3a)²⁹. The structural details of the steps linking IP₃ binding to opening of the pore remain largely speculative, but it seems clear that residues within the extreme N-terminus that form the so-called suppressor domain (SD, residues 1-223) are essential⁴. Two points are important for the method described herein. First, the IBC expressed as a soluble protein in bacteria recognises IP₃ and related ligands with the same specificity as native IP₃R^{4,30}. Second, the conformational changes initiated by IP₃ pass from the IBC via the SD to the pore, allowing the initial activation process to be examined in a soluble, bacterially expressed N-terminal fragment of the IP₃R⁴. We use both IBC and NT fragments to demonstrate the utility of FP for analyses of ligand binding¹⁸.

Experimental design

Protocol overview—The protocol is divided into two parts: (i) preparation of protein and (ii) FP measurements and their analysis (Fig. 4). The first part includes cloning, expression of protein in bacteria, protein purification, and quantification of functional binding sites.

Optimization—The steps must be optimised for each protein and fluorescent ligand. Before scaling-up protein expression methods, pilot experiments are recommended to optimise the choice of purification strategy (which may require engineered tags), conditions for protein expression (growth and induction temperatures, incubation times, choice of plasmid and bacteria, etc), and the final conditions for purification of functional protein in sufficient yield. For expression of N-terminal fragments of IP₃R, we began with a published protocol²² and then introduced some modifications⁴. Similar adaptations of existing protocols are likely to provide the most economical route to expression of other proteins for FP analysis. Our purification strategy used a glutathione-S-transferase (GST) tag provided by a pGEX-6P-2 vector and glutathione sepharose 4B beads for affinity purification. The tag is then removed by cleavage at a PreScission cleavage site. For other proteins, a variety of commercially available tags (e.g. poly-His, biotin, etc) and cleavage sites (e.g. thrombin, enterokinase, etc) offer many opportunities to optimise purification strategies. In deciding between tags and cleavage sites, it is important to consider whether they affect protein expression or function, and whether there are any possible endogenous sites for the cleavage enzyme. The FP assay then requires quantitative measurement of functional ligand-binding sites. After purification, the protein concentration must be determined using a standard assay, for example the Bradford³¹ or bicinchoninic acid assays³² or their many convenient

commercial derivatives. If Western blot and silver-staining analyses identify only a single band, the total protein concentration defines the concentration of the protein of interest. However, in bacterial expression systems there are often several smaller fragments and even if these are absent some of the full-length protein may not be functional. It is therefore essential to quantify directly the concentration of *functional* protein. This is best achieved with a conventional radioligand binding assay, using, for example, centrifugation to separate bound from free radioligand after precipitation of the soluble protein⁴. This definitive measure of the concentration of functional binding sites (in mol ml⁻¹) is essential for FP analyses (Boxes 1 and 2). It is worth comparing this measurement with quantification of silver-stained gels to estimate the fraction of the appropriately sized protein that is functional. There are many options for quantifying bands within gels: we use GeneSnap image acquisition software from Syngene, but ImageJ software is a freely available alternative (<http://rsbweb.nih.gov/ij/>). From the total protein concentration of the sample (in mg ml⁻¹) and quantification of the relative amount of protein within the band of appropriate size (67 kDa for the NT fragment of IP₃R) on a silver-stained gel (in %), the concentration of the 67-kDa protein in the sample can be calculated (mg ml⁻¹). Because there is one IP₃-binding site in each NT fragment, this concentration of the 67-kDa protein (mg ml⁻¹) can be compared with the concentration of IP₃-binding sites in the same sample (nmol mg⁻¹) and with the theoretical density of IP₃-binding sites if each were functional (~15 nmol mg⁻¹). From these calculations, the fraction of the protein within the 67-kDa band that retains a functional IP₃-binding site can be estimated. This provides an index of the quality of the protein preparation. For other proteins too it is essential to develop methods to quantify the concentration of functional protein, and ideally to assess its purity.

The FP assay needs to be optimised for each protein-ligand interaction (Box 3). First, it is necessary to assess whether the fluorescence of the ligand changes when it binds to the protein. It is essential for FP analyses that only the rate of depolarization, but not the fluorescence intensity, changes after binding (Box 2). If binding affects the fluorescent properties of the fluorophore, the latter should be conjugated through another position, or use of another fluorescent ligand and/or protein fragment should be explored. Second, the optimal concentration of fluorescent ligand for an FP assay needs to be determined. This can be achieved by measuring *A* of serially diluted concentrations of fluorescent ligand and choosing the lowest concentration (to keep it below its *K_D*) that allows *A* to be measured reliably free of contributions from background fluorescence (Box 3). Third, it is necessary to establish whether high protein concentrations (independent of their ability to bind ligand) interfere with detection of the polarized light (by absorption, for example). The assay is therefore performed under conditions where a large excess of unlabelled ligand prevents binding of the fluorescently labelled ligand to the protein. If any attenuation of detected light is the same in both planes (*I_{||}* and *I_⊥*), it will not affect the measurement of *A*. Otherwise a correction will be required. These optimisation steps are described in detail in Box 3.

Saturation and competition binding analyses—FP can be used for saturation and competition binding analyses, and so measure affinities for both a fluorescent ligand and unlabelled competing ligands. Both analyses use a fixed concentration of fluorescent ligand. Saturation analysis requires addition of increasing concentrations of protein (ideally covering a range >100-fold either side of the *K_D*), while competition analysis requires addition of increasing concentrations of competing ligand (again, ideally covering a range >100-fold either side of the *K_D*) to a fixed concentrations of protein and fluorescent ligand. The protein concentration should be as low as possible to avoid wasting precious material, but high enough to ensure that most fluorescent ligand (*A* tending to *A_{D*R}*, Box 1) is bound before addition of competing ligand. This maximises the dynamic range of the competition assay. In practice, a protein concentration that gives *A* greater than 50 % of *A_{D*R}* is a

reasonable target (Box 2). The time taken for incubations to reach equilibrium depends on the protein and the ligand, their concentrations and the temperature; it needs to be determined for each set of conditions. Further details of both types of FP assay are provided in **Boxes 1 and 2**.

Equipment requirements—FP measurements require a microplate reader capable of resolving fluorescence polarization. Suitable equipment is available from several suppliers including BMG Labtech (Offenberg, Germany), BioTek (Potton, U. K.) and Berthold Technologies (Harpending, U. K.). Most companies offer multimode microplate readers, which can be used for additional applications (e.g. luminescence, time-resolved fluorescence, etc). For FP measurements, samples are excited with plane polarized light (horizontally or vertically, depending on the equipment) at the excitation wavelength of the fluorophore. Emitted light is simultaneously measured in the parallel ($I_{||}$) and perpendicular ($I_{\perp}$) planes relative to the excitation source (Fig. 2b). Most instruments provide direct readouts of both A and polarization. We use the Pherastar (BMG Labtech). The choice of excitation and emission filters and dichroic mirror is dictated by the excitation and emission spectra of the fluorophore. The intensity of the light detected by the two PMTs is used to determine A (eqn 5). The default FP setting for the Pherastar is ‘plate-mode’, in which each cycle reads the entire plate (rather than a single well). The direction in which the plate is read (left-to-right, etc) is programmable and selected to best match the layout of samples in the 96-well plate (we use direction 11). The Pherastar moves the plate to align each well with the light-path before each reading; a programmable ‘positioning delay’ (minimum 300 ms) allows this to be adjusted to ensure fluid has settled before data collection (we set the delay to 1 s). The optimal ‘focal height’ at which readings are taken depends on the microplates and volumes of fluid. The automatic adjustment (recommended) reads samples at various heights to determine the optimal height for the maximal signal. We read the entire plate three times for each assay, with no lag between cycles (if appropriate, it is possible to program an interval between cycles) (Step 47). Each cycle averages the signal from a programmed number of flashes from the light source: more flashes increase accuracy, but at the expense of longer read-times. We choose 300 flashes per cycle (Step 47). The gain on each PMT must be adjusted to avoid saturation and to balance their sensitivities. If A_{D*} is known, the gains are adjusted to give the expected A_{D*} using a sample with the highest $[D^*]$. In practise, the Pherastar uses polarization (P , see Introduction) rather than A : for fluorescein, $P = 0.035$, and for FITC-IP₃ it is 0.025 (Ref.¹⁸). These values are set as the target values during the automated gain adjustment. The built-in temperature-controlled chamber of the Pherastar can maintain temperatures from about 5 °C above ambient to 45 °C. For lower temperatures, the entire apparatus must be housed in a temperature-controlled cabinet.

Results analysis—A spreadsheet (e.g., Excel), which may initially take 1-2 h to prepare, provides the most economical means of preparing results for analysis. Results can then be imported directly from the equipment and analysed quickly. We use GraphPad Prism version 5 to fit results to Hill equations, but other curve-fitting program can also be used (e.g., MATLAB, Origin, etc).

MATERIALS

REAGENTS

- 1,4-piperazinediethanesulfonic acid (PIPES) (Cat. No. P1851, Sigma)
- 2-mercaptoethanol (Cat. No. 31350-010, GIBCO)

- Adenophostin A (Fig. 3b) was synthesised as described in ref.³³. CRITICAL: It is also available from Calbiochem (Cat. No. 11550)
- Agar standard quality (Cat. No. 05040, Sigma)
- Agarose (Cat. No. A9539, Sigma)
- Aluminium foil
- Ampicillin sodium salt (Cat. No. A0104, Melford Laboratories)
- Anti-glutathione-S-transferase (GST) antibody produced in rabbit (Cat. No. G7781, Sigma)
- Bovine γ -globulin (Cat. No. 345876, Calbiochem)
- DC protein assay kit (Cat. No. 500-0116, BioRad Laboratories)
- DL-dithiothreitol (DTT) solution (Cat. No. 43816, Sigma)
- D-*myo*-inositol-1,4,5-trisphosphate, hexapotassium salt (IP₃) (Fig. 3b) (Cat. No. ALX-307-007, Alexis Biochemicals)
- EcoScint A scintillation solution (Cat. No. LS-273, National Diagnostics)
- Efficient biotinylation *E. coli* K12 Strain AVB100 (Cat. No. AVB100, Avidity) or BL21(DE3) competent cells (Cat. No. C6000-03, Invitrogen)
- Empty disposable PD-10 columns (Cat. No. 17-0435-01, GE Healthcare)
- Ethidium bromide (Cat. No. E7637, Sigma)

! **CAUTION** Ethidium bromide is a mutagen and suspected carcinogen. Use gloves, avoid contamination and contact with skin.

- Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) (Cat. No. E0396, Sigma)
- Ethylenediaminetetraacetic acid (EDTA) (Cat. No. 431788, Sigma)
- FITC-IP₃ (D-2-*O*-(2-(3-(5-fluoresceinyl)thioureido)ethyl)-*myo*-inositol 1,4,5-trisphosphate (Fig. 3b) was synthesised and purified as described in ref.¹⁸
- Glutathione sepharose 4B beads (Cat. No. 17-0756-01, GE Healthcare)
- Goat anti-rabbit IgG-HRP (Cat. No. sc-2004, Santa Cruz Biotechnology, Inc.)
- Half-area, black, round-bottom, polystyrene microplates (Cat. No. 82050-646; Greiner)

▲ **CRITICAL** Optimal choice of microplates is essential. They should be black to reduce cross-talk between wells; half-area to minimise reaction volume; polystyrene to minimise non-specific binding; round-bottom for optimal mixing. Depending on the light path of the optical system (Fig. 2b), the bottom should be either black or transparent

- Inositol 1,4,5-trisphosphate, D-[inositol-1-³H(N)] (³H-IP₃, 681 GBq/mmol) (Cat. No. NET911005UC, Perkin-Elmer)
- InstantBlue Coomassie stain (Cat. No. ISB01L, Expedeon)
- Isopropyl- β -D-thiogalactoside, dioxin-free (IPTG) (Cat. No. IPTG025, Formedium)
- LB agar Miller (Cat. No. LMM0202, Formedium)
- LB broth Miller (Cat. No. LMM0102, Formedium)

- Liquid nitrogen
- Lysozyme chloride from chicken egg white (Cat. No. 9066-59-5, Sigma)
- MagicMark western protein standard (Cat. No. LC5600, Invitrogen)
- NuPAGE MOPS SDS running buffer (20x) (Cat. No. NP0001, Invitrogen)
- NuPAGE novex 4-12% bis-tris gel 1 mm, 12-well (Cat. No. NP0322BOX, Invitrogen)
- Oligonucleotide primers (Invitrogen). CRITICAL: Custom DNA primer synthesis is available from many other suppliers.
- pGEX-6P-2 plasmid vector (Cat. No. 27-4598-01, GE Healthcare)
- Pierce silverSNAP stain kit (Cat. No. 24612, Thermo Scientific)
- Pipette tips for liquid-handling system (e.g. 96-racked, 200 μ l, low-retention tips, Cat. No. 5030075C, Capp)
- Platinum *Pfx* DNA polymerase (Cat. No. 11708-013, Invitrogen)
- Poly(ethylene glycol)8000 (PEG 8000) (Cat. No. P2159, Sigma)
- Pop-culture (Cat. No. M00059238, Novagen)
- Precision plus protein kaleidoscope standards (Cat. No. 161-0375, BioRad)
- PreScission protease (Cat. No. 27-0843-01, GE Healthcare)
- Protease inhibitor cocktail tablets complete mini (Cat. No. 11836153001, Roche)
- QIAprep spin miniprep kit (Cat. No. 27104, Qiagen)
- QuikChange II XL site-directed mutagenesis kit (Cat. No. 200521, Agilent, Stratagene Products)
- RNase solution (Cat. No. R6148-1.5ml, Sigma)
- Sterile petri dishes (10 cm diameter) (Cat. No.633194, Greiner)
- Subcloning efficiency DH5 α chemically competent *E. coli* cells (Cat. No. 18265-017, Invitrogen)
- T4 DNA ligase (Cat. No. M0202L, New England Biolabs)
- Tris acetate-EDTA buffer concentrate (50x) (Cat. No. 67996, Sigma)
- Tris-base (Cat. No. BPE 152-1, Fisher Scientific)

EQUIPMENT

Most equipment is likely to be available in any well-equipped lab. The exceptions are the automated liquid-handling system (which is not essential) and a plate-reader capable of resolving fluorescence polarization (FP) (Fig. 2b).

- 8 or 12-channel pipettes (e.g., Pipetman ultra multi-channel: F21040 (8 $\times$ 20 μ l), F21041 (12 $\times$ 20 μ l), F21042 (8 $\times$ 300 μ l), F21043 (12 $\times$ 300 μ l), Gilson)
- Autoclave (e.g., Crystal 200, Rodwell Scientific Instruments)
- Bench-top centrifuge for 96-well plates (e.g., ALC PK120 plate centrifuge, DJB Labcare, UK)
- Curve-fitting software (e.g., GraphPad Prism 5, GraphPad Software, Inc.)

- End-over-end rotation apparatus (e.g., Rotospin-test tube totator, Tarsons)
- iBlot gel-transfer device (Cat. No. IB1001, Invitrogen)
- iBlot transfer stack, PVDF mini (Cat. No. IB4010-02, Invitrogen)
- Liquid scintillation counter (e.g., LS 6500 scintillation counter, Beckman)
- Liquid-handling system (e.g., CAS-1200, Corbett Robotics UK Limited). This is not essential
- pH meter (e.g., routine pH electrode and meter, Mettler Toledo)
- Plate reader capable of FP measurements (e.g., Pherastar, BMG Labtech) (for further details of equipment requirements see Fig. 2b and Introduction)
- Power supply for electrophoresis and transfer device (e.g., PowerPac basic power supply, BioRad)
- Sonicator (e.g., Transonic T420 bath, CamLab)
- Temperature-controlled cabinet (e.g., Liebherr Profi line, Jencons)
- Temperature-controlled orbital shaker (e.g., Innova 4230 refrigerated benchtop incubator shaker, New Brunswick Scientific)
- Thermal cycler (e.g., PTC-150 Minicycler, MJ Research, GRI)
- Ultracentrifuge and rotor type 50.2 Ti (e.g., Optima L-100K, Beckman Coulter)
- UV transilluminator (e.g., UV transilluminator 2000, BioRad)
- XCell *SureLock* mini-cell (Cat. No. EI0001, Invitrogen)

REAGENTS SETUP

Luria Bertani (LB) medium—Dissolve 25 g of LB-broth Miller in ~800 ml de-ionized water (dH₂O). Add dH₂O to final volume of 1 l. Dispense into media bottles and autoclave to sterilise. Sterile media can be stored for months at 20-25 °C.

Ethidium bromide-stained 1% agarose gel—Add 2.5 g of agarose to 250 ml of tris acetate-EDTA buffer. Swirl and microwave for 1 min until the agarose dissolves completely. Allow the mixture to cool to ~20 °C and then add ethidium bromide (final 0.5 µg ml⁻¹). Pour into a gel tray and allow to solidify (~30 min) before use.

LB agar plates—Add 40 g of LB-agar Miller to 1 l of dH₂O and autoclave. Allow LB agar to cool to ~50 °C and add ampicillin (final 100 µg ml⁻¹). Dispense into sterile Petri dishes (10-cm diameter) and allow to set at ~20 °C. Stable stored at 4 °C for months.

Tris/EDTA medium (TEM)—50 mM Tris-base, 1 mM EDTA, pH 8.3. Stable stored at 4 °C for months.

Phosphate-buffered saline (PBS)—140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3. Stable stored at 4 °C for months.

Ca²⁺-free cytosol-like medium (Ca²⁺-free CLM)—140 mM KCl, 20 mM NaCl, 2 mM MgCl₂, 1 mM EGTA and 20 mM PIPES, pH 7.0. Stable stored at 4 °C for months.

▲ **CRITICAL** Careful correction of pH at the final incubation temperature is critical because the affinity of EGTA for Ca^{2+} , and the affinity of IP_3 for IP_3R are each affected by pH and temperature.

PROCEDURE

Cloning of N-terminal fragments of IP_3R • **TIMING 7 d—1** | Use Platinum *Pfx* DNA polymerase (or other available thermostable DNA polymerase) to amplify N-terminal fragments of rat $\text{IP}_3\text{R1}$. As template use the full-length rat type 1 IP_3R clone lacking the S1 splice site with primers P1 and P2 for the NT (residues 1-604) and P2 and P3 for the IBC (residues 224-604). The sequences of the primers are listed in Table 3. The fragments are numbered with reference to the full-length (S1+, i.e. with the S1 splice site) rat $\text{IP}_3\text{R1}$ (GenBank accession number: GQ233032.1). For each set of primers, set up the reaction as follows:

Component	Volume	Final amount
10x <i>Pfx</i> amplification buffer	5 μl	1x
10 mM dNTP mixture	1.5 μl	0.3 mM each
50 mM MgSO_4	1 μl	1 mM
Primer mix (10 μM each)	1.5 μl	0.3 μM each
Template DNA (200 ng)	1 μl	200 ng
Platinum <i>Pfx</i> DNA polymerase	0.4 μl	1 unit
Autoclaved dH_2O	to 50 μl	

section]

2 | Run PCR reaction in a thermocycler with the following conditions:

Segment	Cycles	Temp	Time
1	1	94 °C	5 min
2	35	94 °C	50 s
		55 °C	1 min
		68 °C	1 min/kb of DNA
3	1	68 °C	10 min

3 | Resolve PCR products (from Step 2) on an ethidium bromide-stained 1 % agarose gel. Electrophorese at 150 V until adequate separation of the PCR bands is achieved. Visualise with a UV-transilluminator.

! **CAUTION** Ethidium bromide is a mutagen and suspected carcinogen. Use gloves, avoid contamination and contact with skin.

4 | Ligate the PCR products into linearised pGEX-6P-2 vectors as *Bam*HI/*Xho*I fragments to give pGEX-NT and pGEX-IBC: to each reaction add 2 μl of reaction buffer (10x), 300 ng of plasmid vector, enough insert to maintain a molar insert:vector ratio of 3:1, 1 μl T4 DNA ligase, and autoclaved dH_2O to a final volume of 20 μl . Incubate 1 h at 22 °C.

5 | Transform the ligation reactions (from Step 4) into competent *E. coli* strain DH5- α cells (or other suitable competent cells) by a standard heat-shock method³⁴.

6 | Plate transformed cells on LB-agar plates containing ampicillin (final 100 $\mu\text{g ml}^{-1}$) and grow for ~12 h at 37 °C to select for ampicillin-resistant clones.

7 | Pick colonies (~10 per plate) and grow each in 2 ml of LB containing ampicillin (final 100 $\mu\text{g ml}^{-1}$) for ~12 h at 37 °C. Pellet cells by centrifugation (6000 *g* for 5 min) and purify plasmid DNA using a standard miniprep plasmid purification kit (e.g., QIAprep spin miniprep kit), following manufacturer's instructions.

8 | Verify identity of the purified plasmid DNA (from Step 7) by restriction enzyme digestion. For each plasmid, add 1 μg of plasmid DNA, 1 μl of 10x buffer, 0.5 μl of each restriction enzyme and dH₂O to a final volume of 10 μl . The same restriction enzymes used for cloning can be used for digestion. Incubate for 1 h at 37 °C. Run digested products in ethidium bromide-stained 1 % agarose gel to check for bands of appropriate size. Confirm sequences of all constructs by DNA sequencing.

! CAUTION Ethidium bromide is a mutagen and suspected carcinogen. Use gloves, avoid contamination and contact with skin.

9 | Use QuikChange II XL site-directed mutagenesis kit to insert the S1 splice site (residues 318-332) into the pGEX-IBC construct (from Step 8). Set up each reaction exactly as described in Step 1, using the purified plasmid DNA from Step 8 as template DNA and primers P4 and P5 (Table 3).

10 | Run PCR reaction in a thermocycler with the following conditions:

Segment	Cycles	Temp	Time
1	1	95 °C	1 min
2	18	95 °C	50 s
		60 °C	50 s
		68 °C	1 min/kb of DNA
3	1	68 °C	7 min

11 | Add 1 μl of the *Dpn* I restriction enzyme to the amplification reaction (from Step 10). Mix and incubate at 37 °C for 1 h to digest the parental DNA.

12 | Transform 2 μl of the digested product into XL10-Gold ultracompetent cells provided with the kit, following the manufacturer's instructions. Plate transformed cells in LB plates containing ampicillin (final 100 $\mu\text{g ml}^{-1}$) and grow for ~12 h at 37 °C to select for ampicillin-resistant clones.

13 | Pick up colonies (~5 per clone) and grow each in 2 ml of LB medium containing ampicillin (final 100 $\mu\text{g ml}^{-1}$) for ~12 h at 37 °C. Pellet cells by centrifugation (6000 *g* for 5 min) and purify plasmid DNA using a standard miniprep plasmid purification kit (e.g., QIAprep spin miniprep kit), following the manufacturer's instructions. Confirm sequences of all constructs by DNA sequencing.

Expression of IP₃R fragments ● **TIMING 3 d—14** | Transform pGEX-NT and pGEX-IBC (obtained in Steps 4-8) into competent *E. coli* AVB101 (or other competent *E. coli* strain such as BL21 D3) by a standard heat-shock method³⁴.

15 | Plate transformed cells on LB agar plates containing ampicillin (100 µg ml⁻¹) and grow overnight at 37 °C.

16 | Inoculate a single colony in 2 ml of LB medium containing ampicillin (final 100 µg ml⁻¹) and incubate in an orbital shaker at 250 r.p.m. for ~12 h at 37 °C.

17 | Inoculate 1 ml of the culture (from Step 16) into 100 ml of LB medium containing ampicillin (final 100 µg ml⁻¹). The cultures can be scaled up to provide the amount of protein required.

18 | Grow the cultures (from Step 17) in an orbital shaker at 22 °C until the OD₆₀₀ reaches about 1-1.5 (after ~7 h). These conditions (notably the incubation temperature of 22 °C, rather than 37 °C used for expression of most proteins) are specifically optimised for expression of N-terminal fragments of IP₃R.

▲ **CRITICAL STEP** It is necessary to optimise growing conditions uniquely for each protein.

? TROUBLESHOOTING

19 | Induce expression of cultures (from Step 18) by adding 100 µl of 0.5 M IPTG (final 0.5 mM). Grow induced cultures at 15 °C with orbital shaking.

▲ **CRITICAL STEP** Expression of IP₃R fragments at reduced temperature is essential to obtain functional protein³⁵.

? TROUBLESHOOTING

20 | 20 h after induction, centrifuge the cells at 6000 *g* for 5 min at 22 °C. Discard the supernatant, wash the pellet with 10 ml of cold PBS by gently pipetting the cells and centrifuge (6000 *g*, 5 min).

21 | Resuspend the pellet in 4.4 ml of ice-cold TEM containing protease inhibitor cocktail (Roche protease inhibitor cocktail tablet complete mini, 1 tablet 10 ml⁻¹). Vortex until the pellet is fully resuspended.

22 | Add 0.5 ml of Pop-Culture (final 10 % vol/vol) and 100 µl of 50 mM 2-mercaptoethanol (final 1 mM) to the resuspended pellet (from Step 21). Incubate the suspension with lysozyme (5 µl of a solution of 100 mg ml⁻¹; final 100 µg ml⁻¹) and RNAase (2.5 µl of a solution of 20 mg ml⁻¹; final 10 µg ml⁻¹) on ice for 30 min.

23 | Sonicate the lysate from Step 22 on ice for 20 s and centrifuge at 30,000 *g* for 60 min at 4 °C. Collect the supernatant.

24 | Save aliquots (~1 ml) of the supernatant (from Step 23) to quantify protein expression by Western blotting (Step 26). The lysate can be frozen in liquid nitrogen before storage at -80 °C.

■ **PAUSE POINT** The lysate is stable at -80 °C for several months.

25 | To allow analysis of protein expression by western blotting, load 1-10 μ l of lysate (from Step 24) per lane, and molecular weight markers onto a NuPage pre-cast 4-12 % gel. Run gel with the XCell *SureLock* mini-cell according to the manufacturer's instructions.

26 | Transfer gel to PVDF membrane using the iBlot gel transfer device according to the manufacturer's instructions. Western blot using anti GST-antibody (1:3000)³⁶ and goat anti-rabbit antibody (1:5000) (Fig. 5a)⁴. The Western blot will reveal whether protein of the expected size has been successfully expressed.

Purification of IP₃R fragments • **TIMING 8 h—27** | If the protein lysate (from Step 23) has been frozen (Step 24), thaw it on ice and then centrifuge at 30,000 *g* for 30 min at 4 °C to clear the supernatant.

▲ **CRITICAL STEP** This clearance step is essential to remove debris.

28 | Gently shake the glutathione sepharose 4B beads to resuspend the medium. Transfer 665 μ l of the slurry (which is ~75 %) to a 50-ml tube. Sediment the slurry by centrifugation at 500 *g* for 5 min. Aspirate the supernatant carefully and discard.

29 | Wash the glutathione sepharose 4B beads by adding 10 ml of cold PBS and separate beads by centrifugation at 500 *g* for 5 min. Aspirate the supernatant carefully and discard. Repeat this wash twice more.

30 | Add 500 μ l of cold PBS to the washed and sedimented glutathione sepharose 4B beads (from Step 29) (to give a 50 % slurry) and add 50 ml of the cleared lysate (from Step 23 or Step 27) to the 50 % slurry and mix by gentle inversion.

31 | Incubate the mixture from Step 30 for 30 min at ~22 °C with gentle (~6 r.p.m.) end-over-end rotation. Pour the mixture into an empty PD-10 column. Tap the column and allow the beads to settle.

▲ **CRITICAL STEP** The efficiency of binding to glutathione sepharose 4B beads can differ considerably between different GST-tagged proteins. Optimal incubation conditions need to be determined for each protein.

▲ **CRITICAL STEP** All procedures hereafter should be performed at 4 °C to minimise protein degradation.

? TROUBLESHOOTING

32 | Open the column outlet and allow the column to drain. Save an aliquot (~50 μ l) of the flow-through for analysis of non-retained proteins by electrophoresis and Coomassie staining (Step 40).

33 | Close the column outlet and wash the drained beads (from Step 32) by adding 10 ml of Ca²⁺-free CLM containing DTT (final 1 mM) to the column. Incubate for 5 min with gentle end-over-end rotation. Open the column outlet and allow the column to drain. Save an aliquot (~50 μ l) of the flow-through for analysis by electrophoresis and Coomassie staining (Step 40).

▲ **CRITICAL STEP** Washing the glutathione sepharose 4B beads after protein binding is important to ensure purity of N-terminally tagged GSTs-fragments.

34 | Repeat wash (Step 33) 4 times. Save aliquots (~50 μ l) from each wash step for analysis by electrophoresis and Coomassie staining (Step 40).

▲ **CRITICAL STEP** After the final wash, ensure the column drains completely to avoid dilution of the final eluate (Step 36).

35 | Prepare PreScission protease mix by adding 40 μ l (80 units) of PreScission protease to 460 μ l of cold Ca^{2+} -free CLM supplemented with 1 mM DTT. Load the PreScission protease mixture onto the column from Step 34.

? TROUBLESHOOTING

36 | Seal the column and incubate with gentle end-over-end rotation for ~12 h at 4 °C.

▲ **CRITICAL STEP** The optimal conditions for cleavage (e.g., amount of enzyme, time, and temperature) need to be determined uniquely for each protein in pilot reactions.

37 | Open the column outlet and collect the eluate, which contains purified IP₃R fragments. PreScission enzyme is GST-tagged, so it remains bound to the column. Save aliquots (~50 μ l) for analysis by electrophoresis and Coomassie staining (Step 40) and quantification of IP₃-binding sites (Steps 41-45).

38 | Add 500 μ l of Ca^{2+} -free CLM supplemented with 1 mM DTT to the column and rotate for 5 min at 4 °C. Collect the eluate, which also contains purified IP₃R fragments. Save aliquots (~50 μ l) for analysis by electrophoresis and Coomassie staining (Step 40) and quantification of IP₃-binding sites (Steps 41-45).

39 | Repeat Step 38.

40 | Load 10-20 μ l of each fraction (Steps 32-34 and 37-39) per lane, and molecular weight markers onto a NuPage pre-cast 4-12 % gel. Run gel with the XCell *SureLock* mini-cell, and stain gel with Coomassie according to the manufacturers' instructions. Check the content of purified protein in each eluate fraction (from Steps 37-39) and pool appropriate fractions. To avoid excessive dilution of the protein, pool only fractions for which the intensity of the Coomassie-stained band is >25 % that of the first fraction. This is the 'purified protein'. Aliquot into small volumes (~100 μ l) and save aliquots for quantification of IP₃-binding sites (Steps 41-45). Freeze remaining aliquots in liquid nitrogen for storage at -80 °C.

■ **PAUSE POINT** The purified protein is stable at -80 °C for several months.

Quantification of the concentration of IP₃-binding sites • TIMING 2 d—41 |

Determine accurately the total protein concentration in the 'purified protein' sample (from Step 40) using the D_C protein assay kit (or other standard protein assay), according to manufacturer's instructions.

42 | Load 1-10 μ g per lane (at least 2 lanes per protein) of purified protein (from Step 40) and molecular weight markers onto two NuPage pre-cast 4-12 % gels. Run both gels with the XCell *SureLock* mini-cell, following the manufacturer's instructions

43 | Perform silver-staining of one gel according to the manufacturer's instructions (Fig. 5a). Transfer the other gel to a PVDF membrane using the iBlot system following the manufacturer's instructions and Western blot with appropriate antisera. We use rabbit antisera (1:1000) raised to peptides corresponding to residues 62-75³⁷ or 326-343 (the SI

splice site)⁴ of IP₃R1 for NT and IBC fragments, respectively. The secondary antibody is HRP-conjugated goat anti-rabbit antibody (1:5000) (Fig. 5a).

▲ **CRITICAL STEP** If the ‘purified protein’ appears as a single band in a silver-stained gel and a Western blot (Step 43) proceed directly to Step 45. It is more likely, however, that the preparation contains the IP₃R fragment contaminated by other minor protein bands. Step 44 is then required to define exactly the concentration of IP₃R fragment within the preparation. It is essential to know precisely the concentration of functional ligand-binding sites for FP analyses (Box 1).

? TROUBLESHOOTING

44 | Quantify the intensity of all the bands in each lane for each protein in both silver-stained gels and western blots. Calculate the percentage of total protein (from the silver-stained gel) present in the band corresponding to the NT (~67 kDa) or IBC (~43 kDa).

? TROUBLESHOOTING

45 | Determine the K_D and density of binding sites (B_{max}) from the same ‘purified protein’ sample (from Step 40) using conventional radioligand binding assays with ³H-IP₃ as described in refs. ^{4,18} (Fig. 5b). Express the concentration of the purified protein as a concentration of binding sites (mol ml⁻¹).

▲ **CRITICAL STEP** Step 45 provides the definitive measure of the concentration of functional IP₃-binding sites (in mol ml⁻¹), which is essential for FP analyses. Inaccurate determination of this concentration will undermine the quantitative FP analysis (Box 1).

Preparation of plates for FP assays ● **TIMING 40 min—46** | This step can be performed using option A for equilibrium saturation binding assays or option B for equilibrium competition binding assays:

A. Preparation of plates for FP equilibrium saturation binding assays:

- i. Load a half-area, black, round-bottom, polystyrene, 96-well plate with duplicate 50- μ l samples of the following in Ca²⁺-free CLM (an example of a plate layout is shown in Fig. 6):
 - Serially diluted NT (15 dilutions from Step 40, final concentration 1-300 nM; labelled R1-R15 in Fig. 6) and FITC-IP₃ (final concentration 0.5 nM); columns A-D in Fig. 6). This allows A_M to be measured for each concentration of NT, and A_{D^*R} (the anisotropy of bound D*) to be determined from the well with the highest (saturating) concentration of NT (Box 1).
 - An identical set of serially diluted NT (final 1-300 nM) and FITC-IP₃ (final 0.5 nM), but supplemented with a saturating concentration of IP₃ (final 10 μ M) (columns E-H in Fig. 6). This ensures that only non-specific binding of FITC-IP₃ persists, allowing A_I (and thereby A_{NS}) to be determined at each concentration of NT (Box 1).
 - A third identical set of serially diluted NT (final 1-300 nM), but without FITC-IP₃. This allows background fluorescence ($I_{||}$ and $I_{\perp}$) at each concentration of NT to be determined (columns I-L in Fig. 6). Where these values are significant, they should be subtracted from the equivalent measurements with FITC-IP₃ present before computing A (Step 50).

- FITC-IP₃ (final 0.5 nM) alone to allow A_{D^*} (the anisotropy of free D^{*}) to be determined (wells C8 and D8 in Fig. 6).

▲ **CRITICAL STEP** Each of these conditions must be included in every plate.

▲ **CRITICAL STEP** The method is described for assays using the NT. For assays with the IBC (which binds most ligands with 10-fold lower K_D than does the NT)⁴, use ~10-fold lower concentrations of binding site.

- Gently shake the plate for 15 min at ~22 °C using an orbital shaker or the orbital shaking mode in the Pherastar plate reader. Centrifuge at 300 *g* for 2 min at ~22 °C.

▲ **CRITICAL STEP** Keep plates and samples wrapped in foil to minimise exposure of fluorescent reagents to light.

- Incubate the plate in the dark at the desired temperature for 20 min to allow equilibrium to be attained. Measure $I_{||}$ and $I_{\perp}$ using the Pherastar plate reader (Steps 47-49). After collection of measurements at one temperature, the instrument and plate can be re-equilibrated to another temperature and the measurements of $I_{||}$ and $I_{\perp}$ repeated on the same samples¹⁸.

▲ **CRITICAL STEP** The time taken for incubations to reach equilibrium must be optimised for each set of conditions.

? TROUBLESHOOTING

B. Preparation of assay plates for FP equilibrium competition binding assays:

- Load a half-area, black, round-bottom, polystyrene, 96-well plate with duplicate 50 μ l samples of the following in Ca²⁺-free CLM:

- Serially diluted competing ligand (e.g., IP₃, final concentrations 3 nM-150 μ M), FITC-IP₃ (final concentration 0.5 nM) and NT (from Step 40, final concentration 80 nM). This allows A_M to be determined at each concentration of competing ligand (Box 2), and A_I to be determined at a saturating concentration of the competing ligand.
- FITC-IP₃ alone (final 0.5 nM) to allow A_{D^*} to be determined.
- FITC-IP₃ (final 0.5 nM) and a saturating concentration of NT (final 300 nM). Under these conditions all FITC-IP₃ is bound, allowing A_{D^*R} to be determined.
- NT (final 80 nM) to allow measurement of background $I_{||}$ and $I_{\perp}$.

▲ **CRITICAL STEP** The concentrations of the competing ligand should ideally cover a range of at least 100-fold either side of its K_D . The method is described for assays using NT. For assays with the IBC use ~10-fold lower concentrations of protein and competing ligand.

▲ **CRITICAL STEP** Protein concentration needs to be optimised for each condition.

- Gently shake the plate for 15 min at ~22 °C using an orbital shaker or the orbital shaking mode in the Pherastar plate reader. Centrifuge at 300 *g* for 2 min at ~22 °C.
- Incubate the plate in the dark at the desired temperature for 20 min to allow equilibrium to be attained. Measure $I_{||}$ and $I_{\perp}$ using the Pherastar plate reader (Steps 47-49).

▲ **CRITICAL STEP** The time taken for incubations to reach equilibrium must be optimised for each set of conditions.

Anisotropy measurements ● TIMING 15 min - 6 h—47 | Open the Pherastar software and select the FP option. Insert the FP 485/520/520 optic module. Set Pherastar options as follows:

- 3 cycles of measurement
- 300 flashes per cycle
- 1 s positioning delay
- reading direction 11 (for details of these settings, see Fig. 2 and equipment requirements in the experimental design section).

48 | Perform auto-adjustment of focal height and gain in the well containing 50 μ l of 0.5 nM FITC-IP₃. Set the 'mP Target' value to 25. The focal height for 50 μ l of solution in a half-area well should be ~6.3.

▲ **CRITICAL STEP** Optimal adjustment of gain and focal height is essential to achieve maximal sensitivity.

49 | Use these settings (Steps 47 and 48) to measure the entire plate (set up as described in Step 46). Samples are excited with horizontally polarized light at 485 nm, and emission is simultaneously measured at 520 nm in the horizontal (i.e. parallel, $I_{||}$) and vertical (i.e. perpendicular, $I_{\perp}$) planes (Fig. 2b).

▲ **CRITICAL STEP** For measurements at different temperatures, the plate reader must be housed in a temperature-controlled cabinet. Allow ~2-3 h for the reaction chamber to cool to 4 °C. Incubate the plate for 20 min at each temperature to allow the reaction to attain equilibrium before measurements. Proceed to Step 50 for analysis.

? TROUBLESHOOTING

Analysis ● TIMING 1 h—50 | For each experimental measurement, subtract the background $I_{||}$ and $I_{\perp}$ due to the presence of the protein alone (Step 46). From eqn 5 calculate A_M using these corrected values of $I_{||}$ and $I_{\perp}$.

51 | Calculate A_I similarly (using eqn 5) from the values of $I_{||}$ and $I_{\perp}$ determined for each protein concentration in the presence of a saturating concentration of competing ligand (IP₃). From A_I and A_{D^*} , calculate A_{NS} (A due to non-specific binding) at each protein concentration using eqns 7 and 8 (Box 1). For each experimental determination of A_M (Step 50) and the corresponding A_{NS} , calculate A_S (A due to specific binding of FITC-IP₃) from eqn 9 (Box 1).

52 | A_S (Steps 50-51) can then be used to calculate the fraction of D^* bound to R using eqn 10 (Box 1) or directly to plot the saturation binding curve (Step 53).

53 | If R_T is sufficient to ensure minimal depletion after binding D^* (Box 1, eqns 11 and 12), plot A_S against R_T ($\sim[R]$) to provide a typical binding curve (Fig. 1a). If R_T is too low to avoid a significant decrease in $[R]$ as D^* binds, $[R]$ must be calculated for each condition (Box 1, eqn 12) before plotting the binding curve. In either case, any suitable curve-fitting program (we use GraphPad Prism version 5) can be used to fit the results to a Hill equation:

$$\alpha = \frac{\left(\frac{[R]}{K_D}\right)^h}{1 + \left(\frac{[R]}{K_D}\right)^h} \quad (13)$$

where α is the fraction of D^* specifically bound, and h is the Hill coefficient. From this we determine the K_D ($K_D^{D^*}$) of the interaction between D^* (FITC-IP₃) and R (NT or IBC).

54 | For equilibrium competition binding measurements (Box 2, Step 46B), calculate A_M and then A_S (Steps 50 and 51). From these A_S values compute the total concentration (I_{50}) of the inhibitor (I) that causes 50 % displacement of specifically bound D^* (Box 2).

55 | From the I_{50} (Step 54), the $K_D^{D^*}$ determined from saturation binding (Step 53) and the known D^*_T and R_T , calculate the *free* concentration of I (IC_{50}) required to displace 50 % of specifically bound D^* (Box 2, eqtn 16) and $[D^*R]$ at the I_{50} ($[D^*R]_{50}$) (Box 2, eqtn 17). The K_D of I for the binding site (K_D^I) is then calculated using eqtn 15.

56 | For experiments performed at different temperatures, the K_D can be calculated from saturation (Step 53) or competition (Step 55) binding experiments at each temperature. Eqtns 3 and 4 define the relationships between K_D , ΔG° , ΔS° and ΔH° . If ΔH° is temperature-independent (i.e. the change in heat capacity, $\Delta C = 0$), a van't Hoff plot ($\ln K_D$ versus $1/T$) can be used to obtain $\Delta H^\circ/R$ from the slope. If $\Delta C \neq 0$ the following equation is used³⁸:

$$\Delta G_T^\circ = \Delta H_{T_o}^\circ + \Delta C_{T_o}^\circ (T - T_o) - T \left[\Delta S_{T_o}^\circ + \Delta C_{T_o}^\circ \ln \left(\frac{T}{T_o} \right) \right] \quad (17)$$

where T is the actual absolute temperature, and T_o is the reference absolute temperature (296 K in our experiments). ΔH° , ΔS° and ΔC° are determined by least-squares curve-fitting of ΔG° versus T .

● **TIMING**—Cloning of N-terminal fragments of IP₃R (Steps 1-13): 7 d

Expression of IP₃R fragments (Steps 14-26): 3 d

Purification of IP₃R fragments (Steps 27-40): 8 h

Quantification of the concentration of IP₃-binding sites (Steps 41-45): 2 d

Preparation of plates for FP assays (Step 46): 40 min

Anisotropy measurements (Steps 47-49): 15 min - 6 h

Analysis (Steps 50-56): 1 h

Optimization of conditions for an FP assay (Box 3): 4 h

TROUBLESHOOTING

Troubleshooting advice can be found in Table 4.

ANTICIPATED RESULTS

Typical yields from expression of N-terminal fragments of IP₃R (IBC and NT) in *E. coli* ($\sim 0.25 \text{ mg l}^{-1}$) are very low because the incubations must be performed at lower than normal incubation temperatures (Step 19)³⁵. For both the NT and IBC, the full-length fragments are expressed with smaller N-terminally tagged products^{4,35}. These shorter fragments are also collected in the eluates (Steps 37-39) after purification with glutathione sepharose 4B beads (Fig. 5a). Western blots and silver-staining of 'purified' NT (from Step 40) show that $\sim 37 \pm 9 \%$ of the *total* protein has the size expected of the NT (Fig. 5a). Similar results were obtained with the IBC¹⁸. Because the relative intensities of the major bands are similar whether identified by silver-staining or western blotting with antibodies to near-N-terminal epitopes (see Step 44), it seems likely that the shorter fragments are C-terminally truncated products. From our knowledge of the minimal requirements for IP₃ binding^{4,18}, it is clear that these truncated products could not bind IP₃. The truncated fragments are unlikely therefore to contribute to specific binding of FITC-IP₃ in the FP assay¹⁸.

In parallel comparisons from the same preparation of 'purified' NT, equilibrium competition and saturation radioligand binding assays using ³H-IP₃ in TEM gave similar estimates of the K_D (1.7 ± 0.2 and $2.2 \pm 0.5 \text{ nM}$, respectively) and B_{max} (1.2 ± 0.1 and $1.5 \pm 0.1 \text{ nmol mg}^{-1}$)¹⁸. Although the K_D of IP₃ for the NT is ~ 40 -fold higher (i.e. lower affinity) in TEM (pH 8.3) than in Ca²⁺-free CLM (pH 7.0), the B_{max} in competition binding assays in Ca²⁺-free CLM matched that obtained in TEM ($1.3 \pm 0.1 \text{ nmol mg}^{-1}$)¹⁸. For our FP analyses, the B_{max} of the 'purified' samples of NT and IBC was determined using equilibrium competition binding experiments in Ca²⁺-free CLM. These means of accurately determining the concentration of IP₃-binding sites are essential for FP analyses (Box 1).

Conventional ³H-IP₃ binding and our FP assay provided similar estimates of the K_D of IP₃, FITC-IP₃ and adenophostin A for the NT and IBC in Ca²⁺-free CLM at 4 °C (Table 5). These values agree also with published results³⁹. Fig. 7a shows an example of a typical equilibrium saturation curve using our FP assay with FITC-IP₃ (final 0.5 nM) and increasing concentrations of purified NT. Fig. 7b shows typical equilibrium competition curves with NT and FITC-IP₃, and either IP₃ or adenophostin A as competing ligands. These results establish that in two different formats (saturation and competition), using two different IP₃R fragments (NT and IBC) and three different ligands (FITC-IP₃, IP₃ and adenophostin A), there is close quantitative agreement in the values of K_D derived from the FP assay and with conventional ³H-IP₃ binding¹⁸. Our analysis provides a robust validation of FP as a reliable means of quantifying ligand interactions with N-terminal fragments of IP₃R.

The non-destructive nature of the FP assay in combination with its ability to detect low-affinity interactions is particularly useful for analyses of the effects of temperature on ligand binding¹⁸. A *single* 96-well plate can be used to measure the K_D (and so ΔG° , eqn 3) at multiple temperatures (allowing computation of ΔH° and ΔS° , eqn 4), thereby considerably reducing both the variability introduced by plate-to-plate differences and use of precious materials (notably purified protein). For example, in a typical 96-well plate format for an equilibrium competition FP binding assay, we obtain K_D values for 3 different ligands from a single plate (with duplicate determinations of 15 concentrations for each ligand) at 6 different temperatures in less than 3 h. Similar experiments using radioligand binding assays would be prohibitively costly, the fast off-rates (k_{-1}) would make them almost impracticable, and they would take days to complete. Before relying on repetitive measurements from the same plate, it is necessary to show that the effects of temperature are fully reversible. In equilibrium competition FP assays, the K_D of IP₃ for the NT ($K_D = 89 \pm 7 \text{ nM}$) at 4 °C was indistinguishable from that obtained when the same plate was incubated

at 37 °C ($K_D = 490 \pm 40$ nM) and then returned to 4 °C ($K_D = 86 \pm 9$ nM). Nor did the K_D of IP₃ for the NT change over the time (~2 h) taken to complete the measurements at different temperatures¹⁸. These results both confirm the stability of the assay and have allowed us to determine ΔH° and ΔS° for binding of different ligands to N-terminal fragments of the IP₃R (Fig. 7c)¹⁸.

BOX 1

COMPARISON OF SATURATION BINDING USING FP AND RADIOLIGANDS

Choice of ligand

Because FP measures fluorescence, any background fluorescence (most likely from protein) must be subtracted (Step 46 and Box 3). Attaching a fluorescent probe to D is more likely than radiolabelling to affect its interaction with R. This needs to be assessed on a case-by-case basis. The K_D for unmodified (non-fluorescent) ligands can, however, be determined by FP using equilibrium *competition* binding experiments (Box 2 and Step46B).

Saturation binding by varying ligand or protein concentrations

Whereas radioligand binding assays vary D^*_T to establish the saturable relationship between $[D^*]$ and RD^* (and so the K_D) (Fig. 1a), FP requires that R_T is varied for the equivalent analysis. This constraint imposes the need to measure R_T accurately (Steps 41-45).

Specific and non-specific binding

A problem common to all analyses of ligand binding is distinguishing saturable binding of D^* to R from low-affinity, non-specific interactions with countless other sites (other proteins, lipids, etc). Because the latter have low affinity, non-specific binding is effectively non-saturable at attainable concentrations of D^* . In resolving A due to non-specific binding, we make two assumptions. First, we assume that A is the same for specifically (A_{D^*R}) and non-specifically bound D^* . Second, we assume (as we do for conventional radioligand binding assays) that non-specific binding increases linearly with $[D^*]$.

A due to non-specific binding (A_{NS}) is determined by measuring A (A_I) at each protein concentration in the presence of a saturating concentration of a ligand (I) that competes with D^* (Step 46) and so displaces all D^* from R (leaving only non-specific binding). But A_I overestimates the non-specific binding that would have been detected in the absence of a competing ligand because in the latter $[D^*]$ is reduced by D^* binding specifically to R. We correct for this by assuming that non-specific binding increases linearly with $[D^*]$:

$$A_{NS} = (A_I - A_{D^*}) (1 - F_B) \quad (7)$$

where A_{D^*} is the A due to free D^* (Step 46); and F_B , the fraction of D^* bound, both specifically and non-specifically ($[D^*]/D^*_T$), is calculated from (eqn 6)⁵¹:

$$F_B = \frac{A_M - A_{D^*}}{A_{D^*R} - A_{D^*}} \quad (8)$$

where A_M is the measured A (Step 50) and A_{D^*R} is A of bound D^* (Step 46). Because non-specific binding varies with the amount of free D^* (eqn 7), it is important to stress that A_{NS} must be separately calculated for each measurement of A in which $[D^*]$ varies. A due to specific binding of D^* (A_S) can then be calculated:

$$A_s = A_M - A_{NS} \quad (9)$$

Distinguishing bound from free ligand

The aim of every binding assay is to quantify the amount of D^* bound to R without perturbing the equilibrium (Fig. 1a). For radioligand binding assays, this presents a challenge because bound and free D^* must be separated (usually by centrifugation or filtration) quickly enough to avoid significantly perturbing the equilibrium (Table 2). Thereafter quantification is straightforward because only bound D^* is trapped on the filter or in the pellet (and where necessary, corrections for trapped volume can easily correct for residual free D^*). The situation is different for FP. Here there is no need to separate bound from free D^* (Fig. 2c), and so no risk of perturbing the equilibrium, but the measured anisotropy (A_M) includes that from free D^* , that bound specifically to R (D^*R) and that bound non-specifically. There are, therefore, two practical issues. First, FP requires that there is no change in the behaviour of the fluorophore (quenching, etc) when D^* binds; D^* must behave as an inert reporter of molecular rotation (Fig. 2c). We need, therefore, to verify that the fluorescence *intensity* of D^* is unaffected by binding (Box 3). Second, we need to relate the measured anisotropy (A_M), from which we derive that due to specific binding (A_s , eqn 9), to the fraction of *specifically* bound D^* :

$$\frac{[D^*R]}{D^*_T} = \frac{A_s - A_{D^*}}{A_{D^*R} - A_{D^*}} \quad (10)$$

A_{D^*R} is determined by measuring A from a sample in which all D^* has bound to R (or by extrapolation of the curve relating A_M to R_T to infinite R_T) (Step 46). Because A is a ratiometric measurement, a useful check of instrumentation and experimental conditions is to verify that A_{D^*} is constant across a range of $[D^*]$ (Box 3)¹⁸.

Changes in $[D^*]$ and $[R]$ as they bind

In order to relate the fraction of D^* bound to R (derived from A_M , eqn 10) to the *free* concentrations of D^* and R (Fig. 1a), we must calculate the relationships between their total and free concentrations for each incubation. Here we encounter a third feature that distinguishes radioligand from FP analyses. With FP it is impossible to use conditions in which $[D^*]$ is unaffected by D^* binding to R, because A changes measurably only when a significant fraction of D^* binds (Fig. 2c). If we were instead to allow $R_T \gg D^*_T$ (analogous to ensuring $D^*_T \gg R_T$ in a radioligand binding assay), then $R_T \approx [R]$ and from eqn 2:

$$\frac{[D^*]}{D^*_T} = \frac{K_D}{K_D + R_T} \quad (11)$$

The K_D can now be determined by measuring the R_T at which $[D^*]/D^*_T = 0.5$. In practise, the purified protein used for FP (R) is likely to be too precious to allow $R_T \gg D^*_T$, and so $[R]$ is likely to decrease as it binds D^* . We must therefore optimise conditions to minimise errors arising from depletion of R (Step 46). If R_T^{50} is the *total* concentration of R required to cause 50 % of $[D^*]$ to be bound, the free $[R]$ under these conditions ($[R]^{50} = K_D$) is reduced by the amount of D^* that has bound, hence:

$$K_D = R_T^{50} - \frac{D_T^*}{2} \quad (12)$$

The aim is to reduce D_T^* to the lowest value compatible with providing adequate fluorescence signals (Box 3). Providing $D_T^* < 10\%$ of K_D , we can assume that $R_T \approx [R]$ (i.e. $K_D \approx R_T^{50}$) and the error will be $< 5\%$. This requirement is equivalent to that imposed in radioligand binding experiments, where we usually aim for $< 10\%$ of D^* being bound.

Anisotropy (A) increases linearly from A_{D^*} to A_{DR^*} as the fraction of bound D^* increases (eqn 6).

The anisotropy (A) of entirely free D^* (A_{D^*}) and of entirely bound D^* (A_{DR^*}) define the maximal dynamic range of an FP assay, within which all experimental values lie. A_M , the measured A of the experimental incubation, includes contributions from free D^* , and D^* bound specifically to R and non-specifically to other components. A_I , measured uniquely for each experimental condition, is the A measured for each assay, but with all specifically bound D^* displaced by a saturating concentration of a competing ligand (I). A_{NS} (anisotropy due to non-specific binding) is calculated from A_I using eqn 7.

BOX 2**EQUILIBRIUM COMPETITION BINDING EXPERIMENTS WITH FP**

Once we have established the K_D of a labelled ligand (D^*) for R (Steps 50-53), we can measure the K_D for any competing unlabelled ligand (I). For FP, a fixed concentration of R and of D^* are chosen to bring A_M close to A_{DR^*} (i.e. most D^* is bound to R). This ensures that the dynamic range of the assay, during which A_M will decrease as D^* is displaced from R by I, is as large as possible. D^*_T should be the lowest concentration compatible with obtaining reliable measures of A (Box 3). The optimal R_T is then a compromise between adding sufficient to bind most D^* and economical use of precious protein. In these equilibrium *competition* binding experiments, the *free* concentration of I needed to displace 50 % of D^* from R must be determined (IC_{50}). Under these conditions, I competes with D^* for binding to R, more I is therefore needed to occupy 50 % of R (IC_{50}) than would be required if I alone were binding to R (K_D). Hence, $IC_{50} > K_D$. The relationship between K_D and IC_{50} comes from considering the fractional occupancy of R by I, when both I and D^* are present²:

$$\frac{[IR]}{R_T} = \frac{\frac{[I]}{K_D^I}}{1 + \frac{[I]}{K_D^I} + \frac{[D^*]}{K_D^{D^*}}}$$

where $[D^*]$ and $[I]$ are the *free* concentrations of D^* and I; and $K_D^{D^*}$ and K_D^I are their equilibrium dissociation constants. From this, derives the Cheng-Prusoff equation⁵²:

$$K_D^I = \frac{IC_{50}}{1 + \frac{[D^*]}{K_D^{D^*}}} \quad (14)$$

Again, it becomes more complicated for FP analyses because the relationships between total and free concentrations are more complicated than for radioligand binding experiments^{53,54}:

$$K_D^I = \frac{[D^*R]_{50} \quad IC_{50} \quad K_D^{D^*}}{(D^*_T R_T) + [D^*R]_{50} (R_T - D^*_T + [D^*R]_{50} - K_D^{D^*})} \quad (15)$$

where IC_{50} (and I_{50}) are the *free* (and *total*) concentrations of competing ligand (I) causing displacement of 50 % of specifically bound D^* , $[D^*R]_{50}$ is the concentration of D^*R at the I_{50} , and $K_D^{D^*}$ is the equilibrium dissociation constant for D^* (Step 55). We need to calculate the relevant *free* concentrations of I, D^* and R. At the I_{50} , 50 % of all R are occupied by I, hence:

$$IC_{50} = I_{50} - \frac{R_T}{2} \quad (16)$$

Because FP measures the fraction of bound D^* ($[D^*]/D^*_T$) (eqn 10), we can calculate $[D^*R]_{50}$ from A_S measured at the I_{50} (A_S^{50}):

$$[D^*R]_{50} = D^* \frac{A_s^{50} - A_{D^*}}{A_{D+R} - A_{D^*}} \quad (17)$$

BOX 3

OPTIMISATION OF AN FP BINDING ASSAY FOR N-TERMINAL FRAGMENTS OF IP₃R ■ TIMING 4 h

Our protocol (Steps 46-56) describes an optimised method for measuring FITC-IP₃ binding to fragments of IP₃R using FP. As a guide to extending the method to other FP assays, the following steps briefly describe some of the key steps¹⁸ that were taken to optimise the protocol.

A | Mix serially diluted NT (from Step 40, final 1-300 nM) and FITC-IP₃ (final 0.5 nM) in Ca²⁺-free CLM. Load duplicate 50-μl samples of each dilution into individual wells of a half-area, black, round-bottom, polystyrene, 96-well plate. Centrifuge the plate at 300 *g* for 2 min at ~22 °C. After 20 min, to allow equilibrium to be attained, measure *total* fluorescence intensity emitted at 520 nm with excitation at 485 nm using any suitable plate reader (we use the Pherastar). Note that in this step, there is no measurement of polarized light.

▲ **CRITICAL STEP** This step assesses whether fluorescence of the ligand (here, FITC-IP₃) changes when it binds to NT.

B | Load duplicate 50-μl samples of serially diluted FITC-IP₃ (final ~0.1 to ~5 nM) in Ca²⁺-free CLM into a half-area, black, round-bottom, polystyrene, 96-well plate. Centrifuge the plate at 300 *g* for 2 min at ~22 °C and measure *I*_{||} and *I*_⊥ (and thereby *A*) using the Pherastar (Steps 47-49).

▲ **CRITICAL STEP** This step aims to find the optimal concentration of fluorescent ligand. Choose the lowest concentration (to keep it below its *K*_D) that allows *A* to be measured reliably free of contributions from background fluorescence. We use 0.5 nM FITC-IP₃ (Step 46).

C | Mix serially diluted purified NT (from Step 40, final 1-300 nM) with FITC-IP₃ (final 0.5 nM) and a saturating concentration of IP₃ (final 10 μM) in Ca²⁺-free CLM. Load duplicate 50-μl samples into a half-area, black, round-bottom, polystyrene, 96-well plate. Gently shake plate at ~22 °C. Centrifuge at 300 *g* for 2 min at ~22 °C. Incubate for 20 min to allow equilibrium to be attained. Measure *I*_{||} and *I*_⊥ (and thereby *A*) using the Pherastar (Steps 47-49).

▲ **CRITICAL STEP** This step aims to establish whether high protein concentrations (independent of their ability to bind ligand) might interfere with detection of the polarized light (by absorption, for example). The assay is therefore performed under conditions where a large excess of unlabelled ligand prevents binding of FITC-IP₃ to the NT. If any attenuation of the detected light is the same in both planes (*I*_{||} and *I*_⊥) (as occurs for FITC-IP₃ and IP₃R fragments)¹⁸, it will not affect the measurement of *A*. Otherwise a correction will be required.

Acknowledgments

This work was supported by the Wellcome Trust (085295) and Biotechnology and Biological Sciences Research Council (BB/H009736). AMR is a fellow of Queens' College, Cambridge. We thank Zhao Ding (University of Cambridge), Barry Potter and Andrew Riley (both University of Bath) for their contributions to our development of FP analyses¹⁸, and Ben Luisi (University of Cambridge) for advice and providing access to equipment during our preliminary FP analyses.

References

1. Foreman, JC.; Johansen, T., editors. Textbook of receptor pharmacology. 2nd ed. CRC Press; Boca Raton, Florida: 2003.
2. Kenakin, TP. A pharmacology primer. Theory, application, and methods. Elsevier; San Diego: 2004.
3. Wyman, J.; Gill, SJ. Binding and linkage. Functional chemistry of biological macromolecules. University Science Books; Mill Valley, California: 1990.
4. Rossi AM, Riley AM, Tovey SC, Rahman T, Dellis O, Taylor EJA, Veresov VG, Potter BVL, Taylor CW. Synthetic partial agonists reveal key steps in IP₃ receptor activation. *Nat. Chem. Biol.* 2009; 5:631–639. [PubMed: 19668195]
5. Keeler, J.; Wothers, P. Chemical structure and reactivity. An integrated approach. Oxford University Press; Oxford: 2008.
6. Atkins, P.; De Paula, J. Elements of physical chemistry. 4th ed. Oxford University Press; Oxford: 2005.
7. Borea PA, Dalpiaz A, Varani K, Gilli P, Gilli G. Can thermodynamic measurements of receptor binding yield information on drug affinity and efficacy? *Biochem. Pharmacol.* 2000; 60:1549–1456. [PubMed: 11077036]
8. Williams DH, Zhou M, Stephens E. Ligand binding energy and enzyme efficiency from reductions in protein dynamics. *J. Mol. Biol.* 2006; 355:760–767. [PubMed: 16325850]
9. Holdgate GA, Ward WH. Measurements of binding thermodynamics in drug discovery. *Drug Disc. Today.* 2005; 10:1543–1550.
10. Weiland GA, Minneman KP, Molinoff PB. Fundamental difference between the molecular interactions of agonists and antagonists with the β -adrenergic receptor. *Nature.* 1979; 281:114–117. [PubMed: 38405]
11. Serdyuk, I.; Zaccaria, N.; Zaccari, J. Methods in molecular biophysics. Structure, dynamics and function. Cambridge University Press; 2007.
12. Weber G. Polarization of the fluorescence of macromolecules. I. Theory and experimental method. *Biochem. J.* 1952; 51:145–155. [PubMed: 14944566]
13. French T, So PT, Weaver DJ Jr, Coelho-Sampaio T, Gratton E, Voss EW Jr, Carrero J. Two-photon fluorescence lifetime imaging microscopy of macrophage-mediated antigen processing. *J. Microsc.* 1997; 185:339–353. [PubMed: 9134740]
14. Owicki JC. Fluorescence polarization and anisotropy in high throughput screening: perspectives and primer. *J. Biomol. Screen.* 2000; 5:297–306. [PubMed: 11080688]
15. Lokesh GL, Rachamalla A, Kumar GD, Natarajan A. High-throughput fluorescence polarization assay to identify small molecule inhibitors of BRCT domains of breast cancer gene 1. *Anal. Biochem.* 2006; 352:135–141. [PubMed: 16500609]
16. Liu Y, Jiang J, Richardson PL, Reddy RD, Johnson DD, Kati WM. A fluorescence polarization-based assay for peptidyl prolyl cis/trans isomerase cyclophilin A. *Anal. Biochem.* 2006; 356:100–107. [PubMed: 16737676]
17. Do EU, Choi G, Shin J, Jung WS, Kim SI. Fluorescence polarization assays for high-throughput screening of neuropeptide FF receptors. *Anal. Biochem.* 2004; 330:156–163. [PubMed: 15183774]
18. Ding Z, Rossi AM, Riley AM, Rahman T, Potter BVL, Taylor CW. Binding of inositol 1,4,5-trisphosphate (IP₃) and adenophostin A to the N-terminal region of the IP₃ receptor: thermodynamic analysis using fluorescence polarization with a novel IP₃ receptor ligand. *Mol. Pharmacol.* 2010; 77:995–1004. [PubMed: 20215561]

19. Duckworth BP, Aldrich CC. Development of a high-throughput fluorescence polarization assay for the discovery of phosphopantetheinyl transferase inhibitors. *Anal. Biochem.* 2010; 403:13–19. [PubMed: 20382102]
20. Zhang R, Mayhood T, Lipari P, Wang Y, Durkin J, Syto R, Gesell J, McNemar C, Windsor W. Fluorescence polarization assay and inhibitor design for MDM2/p53 interaction. *Anal. Biochem.* 2004; 331:138–146. [PubMed: 15246006]
21. Smith DS, Eremin SA. Fluorescence polarization immunoassays and related methods for simple, high-throughput screening of small molecules. *Anal. Bioanal. Chem.* 2008; 391:1499–1507. [PubMed: 18264817]
22. August EM, Patnaude L, Hopkins J, Studts J, Gautschi E, Shrutkowski A, Kronkaitis A, Brown M, Kabcenell A, Rajotte D. Development of a high-throughput assay to measure histidine decarboxylase activity. *J. Biomol. Screen.* 2006; 11:816–821. [PubMed: 16923847]
23. Montagnaro S, Longo M, Mallardo K, Pisanelli G, De Martino L, Fusco G, Baldi L, Pagnini U, Iovane G. Evaluation of a fluorescence polarization assay for the detection of serum antibodies to *Brucella abortus* in water buffalo (*Bubalus bubalis*). *Vet. Immunol. Immunopathol.* 2008; 125:135–142. [PubMed: 18599128]
24. Eglén RM. An overview of high throughput screening at G protein coupled receptors. *Front. Drug Des. & Disc.* 2005; 1:97–115.
25. Kleman-Leyer KM, Klink TA, Kopp AL, Westermeyer TA, Koeff MD, Larson BR, Worzella TJ, Pinchard CA, van de Kar SA, Zaman GJ, Hornberg JJ, Lowery RG. Characterization and optimization of a red-shifted fluorescence polarization ADP detection assay. *Assay Drug Dev. Technol.* 2009; 7:56–67. [PubMed: 19187009]
26. Foskett JK, White C, Cheung KH, Mak DO. Inositol trisphosphate receptor Ca^{2+} release channels. *Physiol. Rev.* 2007; 87:593–658. [PubMed: 17429043]
27. Marchant JS, Parker I. Role of elementary Ca^{2+} puffs in generating repetitive Ca^{2+} oscillations. *EMBO J.* 2001; 20:65–76. [PubMed: 11226156]
28. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 2000; 1:11–21. [PubMed: 11413485]
29. Bosanac I, Alattia J-R, Mal TK, Chan J, Talarico S, Tong FK, Tong KI, Yoshikawa F, Furuichi T, Iwai M, Michikawa T, Mikoshiba K, Ikura M. Structure of the inositol 1,4,5-trisphosphate receptor binding core in complex with its ligand. *Nature.* 2002; 420:696–700. [PubMed: 12442173]
30. Rossi AM, Riley AM, Potter BVL, Taylor CW. Adenophostins: high-affinity agonists of IP_3 receptors. *Curr. Topics Membr.* 2010; 66:209–233.
31. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976; 72:248–254. [PubMed: 942051]
32. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 1985; 150:76–85. [PubMed: 3843705]
33. Borissow CN, Black SJ, Paul M, Tovey SC, Dedos SG, Taylor CW, Potter BVL. Adenophostin A and analogues modified at the adenine moiety: synthesis, conformational analysis and biological activity. *Org. Biomol. Chem.* 2005; 3:245–252. [PubMed: 15632966]
34. Inoue H, Nojima H, Okayama H. High efficiency transformation of *Escherichia coli* with plasmids. *Gene.* 1990; 96:23–28. [PubMed: 2265755]
35. Yoshikawa F, Uchiyama T, Iwasaki H, Tomomori-Satoh C, Tanaka T, Furuichi T, Mikoshiba K. High efficient expression of the functional ligand binding site of the inositol 1,4,5-trisphosphate receptor in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 1999; 257:792–797. [PubMed: 10208862]
36. Stegert MR, Hergovich A, Tamaskovic R, Bichsel SJ, Hemmings BA. Regulation of NDR protein kinase by hydrophobic motif phosphorylation mediated by the mammalian Ste20-like kinase MST3. *Molecular and Cellular Biology.* 2005; 25:11019–11029. [PubMed: 16314523]
37. Cardy TJA, Traynor D, Taylor CW. Differential regulation of types 1 and 3 inositol trisphosphate receptors by cytosolic Ca^{2+} . *Biochem. J.* 1997; 328:785–793. [PubMed: 9396721]

38. Wittmann HJ, Seifert R, Strasser A. Contribution of binding enthalpy and entropy to affinity of antagonist and agonist binding at human and guinea pig histamine H₁-receptor. *Mol. Pharmacol.* 2009; 76:25–37. [PubMed: 19346300]
39. Rossi A, Sureshan KM, Riley AM, Potter BVL, Taylor CW. Selective determinants of inositol 1,4,5-trisphosphate and adenophostin A interactions with type 1 inositol 1,4,5-trisphosphate receptors. *Br. J. Pharmacol.* 2010; 161:1070–1085. [PubMed: 20977457]
40. Owicki JC. Fluorescence polarization and anisotropy in high throughput screening: perspectives and primer. *J. Biomol. Screen.* 2000; 5:297–306. [PubMed: 11080688]
41. de Azevedo WF Jr, Dias R. Experimental approaches to evaluate the thermodynamics of protein-drug interactions. *Current Drug Targets.* 2008; 9:1071–1076. [PubMed: 19128217]
42. McDonnell JM. Surface plasmon resonance: towards an understanding of the mechanisms of biological molecular recognition. *Curr. Op. Chem. Biol.* 2001; 5:572–577.
43. Inoue T, Kikuchi K, Hirose K, Iino M, Nagano T. Synthesis and evaluation of 1-position-modified inositol 1,4,5-trisphosphate analogs. *Bioorg. Med. Chem. Lett.* 1999; 9:1967–1702.
44. Thompson NL, Lieto AM, Allen NW. Recent advances in fluorescence correlation spectroscopy. *Curr. Op. Struct. Biol.* 2002; 12:634–641.
45. Levitt JA, Matthews DR, Ameer-Beg SM, Suhling K. Fluorescence lifetime and polarization-resolved imaging in cell biology. *Cur. Op. Biotechnol.* 2009; 20:28–36.
46. Bosworth N, Towers P. Scintillation proximity assay. *Nature.* 1989; 341:167–168. [PubMed: 2550824]
47. Patel S, Harris A, O'Beirne G, Cook ND, Taylor CW. Kinetic analysis of inositol trisphosphate binding to pure inositol trisphosphate receptors using scintillation proximity assay. *Biochem. Biophys. Res. Commun.* 1996; 221:821–825. [PubMed: 8630045]
48. Axelrod D. Total internal reflection fluorescence microscopy in cell biology. *Traffic.* 2001; 2:764–774. [PubMed: 11733042]
49. Sako Y, Yanagida T. Single-molecule visualization in cell biology. *Nat. Rev. Mol. Cell Biol.* 2003; (Suppl):SS1–SS5. [PubMed: 14587519]
50. Vogel SS, Thaler C, Koushik SV. Fanciful FRET. *Sci STKE.* 2006; 2006:re2. [PubMed: 16622184]
51. Jameson DM, Sawyer WH. Fluorescence anisotropy applied to biomolecular interactions. *Methods Enzymol.* 1995; 246:283–300. [PubMed: 7752928]
52. Cheng Y-C, Prusoff WH. Relationship between the inhibition constant (K_I) and the concentration of inhibitor causing 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* 1973; 22:3099–3108. [PubMed: 4202581]
53. Nikolovska-Coleska Z, Wang R, Fang X, Pan H, Tomita Y, Li P, Roller PP, Krajewski K, Saito NG, Stuckey JA, Wang S. Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. *Anal. Biochem.* 2004; 332:261–273. [PubMed: 15325294]
54. Kenakin, TP. Pharmacologic analysis of drug-receptor interactions. 3rd ed. Lippincott, Williams and Wilkins; New York: 1997.

Figure 1.

Saturable binding of a ligand (D) to its target (R). **(a)** At equilibrium, increasing concentrations of D (expressed relative to its K_D) bind to R until all of the latter has associated to form DR. Note that when $[D] = K_D$, 50 % of R has bound to D. **(b)** Changes in Gibbs free energy are shown for mixtures of D, R and DR (each in their standard states). The standard Gibbs free energy change of the reaction (ΔG° , the difference in the molar Gibbs energies of the reactants and products) is shown and defines ΔG of the (hypothetical) reaction in which 1 mole of R and 1 mole of D combine to form 1 mole of DR (each in their standard states). Because reactions proceed spontaneously in the direction of reduced Gibbs free energy (i.e. yellow $\rightarrow$ blue, but not blue $\rightarrow$ yellow on the plot), the association of D and R proceeds to the minimal Gibbs free energy; this then defines the ratio $[D][R]/[DR]$ at equilibrium (i.e. K_D).

Figure 2.

Ligand binding analysed by fluorescence polarization. (a) If a fluorophore in which the absorbing and emitting dipoles are parallel is excited with plane-polarized light and remains immobile during its fluorescence lifetime, 60 % of the emitted light will remain polarized with respect to the excitation light (i.e. parallel). If the molecule tumbles during its fluorescence lifetime, less than 60 % of the emitted light will be polarized in the parallel plane and more will be detected in each of the perpendicular planes. (b) Typical layout of an FP apparatus used to read microplates. Excitation light (typically a xenon flash lamp) passes through excitation and polarization (horizontal) filters and is then reflected by a dichroic mirror to the sample. Emitted light passes through a dichroic mirror and is then split equally by a beam-splitter, which directs it to photomultiplier tubes (PMT) via horizontal (A) and vertical (B) polarization filters and then emission filters. Further details of equipment are provided in the Equipment requirements section of the Introduction. (c) A small fluorescent ligand (D^*) free in solution is excited with plane polarized light. Its rapid tumbling (due to its small molecular volume) causes emission of depolarized light and a low A (top). When D^* binds R, it increases its effective volume and therefore tumbles more slowly, causing more emitted light to remain polarized in the same plane as the excitation light; A therefore increases (bottom). This allows FP to be used to measure ligand binding to a larger macromolecule.

Figure 3.

Structure of the IP₃ receptor and the ligands used. **(a)** Schematic representation of a single subunit of a tetrameric IP₃R1 showing key regions: the NT (residues 1-604) which comprises the IBC (residues 224-604) and SD (residues 1-223), and 6 transmembrane domains (TMD) **(b)** The crystal structure of the IBC with IP₃ bound (PDB, 1N4K) is shown, highlighting the 2-*O*-atom (arrow on the enlarged view) to which fluorescein 5-isothiocyanate is attached by a short linker to give FITC-IP₃. **(c)** Structures of IP₃, FITC-IP₃ and adenophostin A.

Figure 4. Experimental design flow chart. The protocol is divided into two parts: (i) protein preparation and (ii) anisotropy measurements and analysis.

Figure 5.

Expression, purification and quantification of NT fragments of IP₃R. **(a)** Silver-stained gel and Western blot (WB) showing the NT (~67 kDa, arrows) and smaller contaminating proteins. Molecular weight markers are shown on the left. The relative intensities of the three major bands were similar in WB and after silver-staining, indicating that the smaller proteins probably correspond to C-terminally truncated fragments of the NT. These smaller products are unlikely to bind IP₃ (Refs.^{4,18}). **(b)** The Scatchard plot shows typical results from a saturation binding assay using ³H-IP₃, NT (30 ng per incubation) in TEM. From this plot, the density of binding sites ($B_{\max}$, intercept on the x-axis) and affinity ($-K_A$, slope of the line) can be determined. Although the Scatchard plot conveniently presents binding results, non-linear curve-fitting to a Hill equation (Step 53) provides a more accurate means of determining $B_{\max}$ and K_A . Results, means $\pm$ SEM, $n = 3$, are reproduced from¹⁸ with permission from The American Society for Pharmacology and Experimental Therapeutics.

	A	B	C	D	E	F	G	H	I	J	K	L
1	R1	R1	R9	R9	R1	R1	R9	R9	R1	R1	R9	R9
2	R2	R2	R10	R10	R2	R2	R10	R10	R2	R2	R10	R10
3	R3	R3	R11	R11	R3	R3	R11	R11	R3	R3	R11	R11
4	R4	R4	R12	R12	R4	R4	R12	R12	R4	R4	R12	R12
5	R5	R5	R13	R13	R5	R5	R13	R13	R5	R5	R13	R13
6	R6	R6	R14	R14	R6	R6	R14	R14	R6	R6	R14	R14
7	R7	R7	R15	R15	R7	R7	R15	R15	R7	R7	R15	R15
8	R8	R8	D*	D*	R8	R8			R8	R8		

Figure 6.

Typical 96-well plate layout for the FP saturation binding assay. All wells in columns A-H contain FITC-IP₃ (0.5 nM). Protein dilutions (1 -300 nM) are labelled R1-R15. Columns A-D include no further additions (allowing A_M to be determined); columns E-H also include a saturating concentration of IP₃ (10 μ M, allowing A_{NS} to be determined). Columns I-L include only the serial dilutions of protein (to allow background fluorescence to be measured). Wells C8 and D8 include only FITC-IP₃ (to allow A_{D*} to be determined). Further details in Step 46A. The layout is typical, but it is advisable to vary the layout between experiments to avoid systematic errors in automated pipetting, etc.

Figure 7.

Typical results from FP analysis of equilibrium ligand binding to N-terminal IP₃ receptor fragments **(a)** FP saturation binding assay at 4 °C using FITC-IP₃ (0.5 nM) and the indicated concentrations of NT (Step 53). **(b)** FP competition binding assay using FITC-IP₃ (0.5 nM), NT (80 nM) and the indicated concentrations of either IP₃ or adenophostin A at 4 °C (Step 54) **(c)** van't Hoff plots for IP₃ and adenophostin A binding to the NT (Step 56). Results **(a-c)** are means $\pm$ S.E.M., $n = 3$. The same code applies to panels **b-c**. All FP analyses were performed in Ca²⁺-free CLM. Data reproduced from Ref.¹⁸ with permission from The American Society for Pharmacology and Experimental Therapeutics.

TABLE 1

Definitions and abbreviations

Abbreviation	Definition
α	Fractional occupancy (eqtn 2)
A	Anisotropy (eqtn 5)
A_{D^*R}	A for D^* bound to R
A_{D^*}	A for free D^* (FITC-IP ₃ in our experiments)
A_I	A determined in the presence of a saturating concentration of a competing ligand (IP ₃ in our experiments)
A_M	Measured A (eqtn 6)
A_{NS}	A due to non-specific binding of D^* (eqtn 7)
B_{max}	Maximal number of binding sites
A_S	A due to specific binding
CLM	Cytosol-like medium
[D], [R]	Equilibrium free concentration of D (or R). D^* denotes a radiolabelled or fluorescently tagged version of D
D_T, R_T	Total concentration of D (or R)
dH ₂ O	Deionized water
ΔG°	Standard Gibbs free energy change
ΔH°	Standard enthalpy change
ΔS°	Standard entropy change
FCS	Fluorescence correlation spectroscopy
FITC-IP ₃	D-2-O-(2-(3-(5-fluoresceinyl)thioureido)ethyl)-IP ₃ (Fig. 3b)
FLIM	Fluorescence lifetime imaging microscopy
FRET	Fluorescence resonance energy transfer
FP	Fluorescence polarization
I_{50}, IC_{50}	Total (I_{50}) and free (IC_{50}) concentration of a competing ligand (I) causing 50 % displacement of specifically bound D^* (eqtn 16)
$I_{ }$	Fluorescence intensity measured in the parallel plane
$I_{\perp}$	Fluorescence intensity measured in the perpendicular plane
ITC	Isothermal titration calorimetry
IP ₃	Inositol 1,4,5-trisphosphate (Fig. 3b)
IP ₃ R	Inositol 1,4,5-trisphosphate receptor
IBC	IP ₃ -binding core of IP ₃ receptor (residues 224-604 of IP ₃ R1) (Fig. 3a)
NT	N-terminal domain of IP ₃ receptor (residues 1-604 of IP ₃ R1)
k_{+1}, k_{-1}	Rate constants (eqtn 1)
K_A, K_D	Equilibrium association (K_A) or dissociation (K_D) constant ($K_A = 1/K_D$) (eqtn 1)
ρ	Rotational relaxation time
R	Universal gas constant
SD	Suppressor domain of IP ₃ receptor (residues 1-223 of IP ₃ R1) (Fig. 3a)
SPA	Scintillation proximity assay

Abbreviation	Definition
SPR	Surface plasmon resonance
T	Absolute temperature
TEM	Tris/EDTA medium
TIRFM	Total internal reflection fluorescence microscopy

TABLE 2

Comparison of methods commonly used to measure ligand binding

Method	Principle	Inexpensive reagents	Inexpensive equipment	No separation	Scatchometry	Rate constants	ΔF and ΔS^a	Native ligand	Intact cells	Real-time measurement	Single-molecule resolution	High-throughput	No protein purification	No immobilization
Separation	D* (usually radioactive, but other tags can be used) is incubated with R. At equilibrium, free and bound D* are separated, usually by filtration or centrifugation, and bound D* is quantified (usually by liquid scintillation counting) ⁴ .	x	✓	x	✓	✓	✓	x _a	✓ _b	x	x	x	✓	✓
FP	Fluorescence polarization detects binding of a small fluorescent ligand (D*) to a larger protein using plane-polarized light to detect the change in effective molecular volume ^{33,35} (Fig. 2).	✓	✓	✓	✓	x	✓	x _a	x	✓	x	✓	x	✓
ITC	Isothermal titration calorimetry measures directly the tiny amounts of heat released or absorbed (ΔH) as increasing concentrations of D are added to purified R ⁴¹ .	x _c	x	✓	✓	✓	✓ _d	✓	x	✓	x	x	x	✓
SPR	Surface plasmon resonance detects the increase in size of an immobilised ligand when it binds a larger protein by recording the change in refractive index at the surface of a thin metal film ^{42,43} .	x	x	✓	✓	✓	x	x	x	✓	x	x	✓	x
FCS	By measuring fluorescence intensity from a small volume over short intervals and calculating autocorrelation functions, fluorescence correlation spectroscopy allows rates of translational diffusion to be measured in intact cells. Binding of a small fluorescent ligand to a larger protein can thereby be detected ⁴⁴ .	✓	x	✓	x	✓	x	x	✓	✓	✓	x	✓	✓
FLIM	When a fluorophore associates with another species it can cause the fluorescence lifetime to change, this provides the basis for fluorescence lifetime imaging microscopy ⁴⁵ .	✓	x	✓	x	✓	x	x	✓	✓	x	x	✓	✓
SPA	For scintillation proximity assays, R is immobilised on a bead containing scintillant, which detects radioactive D* only when it comes close to the bead. Only D* bound to immobilised R is thereby detected. The assay is conducted in a liquid scintillation counter ^{46,47} .	x	✓	✓	x	✓	x	x	x _a	x	x	x	✓	x
TIRFM	By illuminating only a very thin field (~100 nm) above a coverslip, total internal reflection microscopy detects only fluorescent molecules within the field. Binding of fluorescent D* to an immobilised R can thereby be detected ^{48,49} .	✓	x	✓	✓	✓	x	x	✓	✓	✓	x	✓	x
FRET	Fluorescence resonance energy transfer is the non-radiative transfer of energy between two fluorophores with overlapping excitation and emission spectra. Because FRET efficiency depends on the separation of the fluorophores, it can be used to monitor association of fluorescently labelled D* and R ⁵⁰ .	x	x	✓	✓	✓	x	x	✓	✓	✓	✓	✓	✓

and x indicate whether the method does () or does not (x) satisfy the criterion.

^aK_D for unlabelled ligands can be determined in equilibrium competition binding assays with labelled ligand.

^bBinding to cell-surface receptors only.

^cCostly to purify the large amounts of protein required.

^dITC is the only method to measure ΔH directly

TABLE 3

Primers used to generate NT and IBC constructs. Primer sequences are 5' to 3'

Primer	Sequence	Step
P1	CGGGATCCATGTCTGACAAAATGTCTAGT	1
P2	CGCGCTCGAGTCACTTTCGGTTGTGTGGA	1
P3	CGGGATCCATGAAATGGAGTAACAAAG	1
P4	ATTACTTGGCAGCAGAGGTAGACCCTGACTTTGAGGAAG AATGCCTGGAGTTTCAGCCCTCAGTGGACCCTGATCAGG	9
P5	GATCAGGGTCCACTGAGGGCTGAAACTCCAGGCATTCTT CCTCAAAGTCAGGGTCTACCTCTGCTGCCAAGTAATGC	9

TABLE 4

Troubleshooting

Step	Problem	Possible reason	Solution
18-19	No protein expression or very low yield.	The conditions (temperature and incubation times) for bacterial growth and/or induction were not optimal.	Run small-scale cultures to determine optimal conditions. For IP ₃ R fragments, incubation at low temperature is essential to obtain functional protein (most other proteins are expressed at much higher yields at 37 °C).
32	GST-tagged protein does not bind to glutathione sepharose beads and appears in the flow-through and washes.	The protein might have been denatured during lysis. The fusion protein may have caused misfolding.	Use milder conditions for lysis. Prepare a lysate of cells transformed with empty pGEX plasmid and check binding to the glutathione sepharose beads under similar conditions. Increase the DTT concentration in the binding buffer. DTT (final 1–10 mM) can increase binding of some GST-tagged proteins to glutathione sepharose beads.
37-39	Too little purified protein in the eluates.	The tag was not cleaved, perhaps due to the presence of protease inhibitors (e.g., >50 mM Tris-HCl, >150 mM NaCl, >1 mM EDTA, >1 mM DTT) during the cleavage reaction. The cleavage conditions were not optimal.	Change the buffer composition to avoid inhibitors. Perform pilot reactions to determine optimal conditions (amount of enzyme, ratio protease/tagged protein and reaction time).
43	Purified protein is not detected by the antibody.	Many commercially available antibodies are useless.	Examine carefully the properties of antibodies before relying on them to define the purity of the 'purified protein'. These characterization steps need to be performed for each antibody and purified protein.
44	Non-linear relationship between amount of protein within a band and quantification of its staining.	Lanes in either the silver-stained gel or western blot were overloaded.	Load a range of protein concentrations (Step 42) to establish that the detected intensity is linearly related to the amount of sample loaded.
46	Reactions have not attained equilibrium before measurements.	The incubation time is too brief for equilibrium to be attained.	Optimise incubation time for each set of conditions by measuring the same plate using Pherastar (Steps 47–49) at regular intervals (i.e. every 5 min) at the appropriate temperature. Follow the increase in <i>A</i> with time, and determine the time at which <i>A</i> reaches a plateau, indicating the approach to equilibrium. Lower concentrations (of ligand and/or protein) will take longer.
	Increasing the temperature affects the measurements.	Where the same plate is incubated at different temperatures, there is a risk of protein damage or, with some assays, degradation or metabolism of the ligand, at elevated temperatures.	Verify that effects of increased temperature on ligand binding are reversed when the plate is returned to the lower temperature ¹⁸ .

TABLE 5
Comparison of results obtained by FP and conventional radioligand binding assays

The K_D (nM) for each ligand determined in Ca^{2+} -free CLM at 4 °C by equilibrium competition binding assays using $^3\text{H-IP}_3$ or by FP is shown as mean $\pm$ S.E.M., $n = 3$. Results are taken from¹⁸ with permission from The American Society for Pharmacology and Experimental Therapeutics.

	IBC		NT	
	$^3\text{H-IP}_3$	FP	$^3\text{H-IP}_3$	FP
FITC- IP_3	2.0 ± 0.2	3.0 ± 0.1	11.8 ± 0.2	12.5 ± 0.6
IP_3	8.7 ± 1.8	9.2 ± 0.8	65 ± 8	95 ± 7
Adenophostin A	0.7 ± 0.1	0.9 ± 0.1	6.2 ± 1.6	8.6 ± 0.2