Analyses of ligand binding to IP_3 receptors using fluorescence polarization

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Running Head

FP analysis of IP_3 binding

i. Summary/Abstract

Fluorescence polarization (FP) can be used to measure binding of a small fluorescent ligand to a larger protein because the ligand rotates more rapidly in its free form than when bound. When excited with plane polarized light, the free fluorescent ligand emits depolarized light, which can be quantified. Upon binding, its rotation is reduced and more of the emitted light remains polarized. This allows FP to be used as a non-destructive assay of ligand binding. Here we describe a fast, high-throughput FP assay to quantify the binding of fluorescently labelled inositol 1,4,5-trisphosphate (IP_3) to N-terminal fragments of the IP_3 receptor. The assay is fast (1-6 hours), it avoids use of radioactive materials and when measurements are performed at different temperatures, it can resolve Gibbs free energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) changes of ligand binding.

ii. Key Words

IP_3 receptor, fluorescence polarization, ligand binding, affinity, thermodynamics.

1. Introduction

Molecular interactions underpin all biological activity. Quantitative analysis of ligand-protein interactions is essential to understanding cellular behaviour. Although several methods have been applied for quantitative analysis of ligand-protein interactions [1], most studies have

used radioligand binding assays. These methods are laborious and time-consuming, they are not amenable to high-throughput analyses, and they use expensive and environmentally unfriendly radioactive materials. A major drawback of radioligand binding assays is the need to separate bound from free ligand, which hampers quantification of low-affinity interactions. Fluorescence polarization (FP) assays provide an alternative approach that avoids these problems. FP measures the change in rotational speed of a fluorescent ligand during its excited lifetime as a consequence of it binding to a larger protein or receptor. A fluorophore is optimally excited (typically in 10^{-15}s) by plane-polarized light in a preferred orientation and then (usually) [2, 3] emits light in the same orientation. If there is no movement of the molecule during the fluorescent lifetime of the fluorophore (typically ns), then 60% of all emitted light would be detected in the same plane as the exciting light (parallel) and 20% in each of the other two planes (perpendicular) (Fig. 1A). But if the molecule rotates (<ns timescales for small molecules), it will no longer be aligned and when the fluorophore emits its light <60% will be aligned with the source (Fig. 1A). When plane-polarized light is used for excitation, then the intensities of the light detected in the perpendicular ($I_{\perp}$) and parallel planes ($I_{\parallel}$) with respect to the excitation source are related to the polarization (*P*) and anisotropy (*A*):

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \text{ (equation } \mathbf{1}\text{)}$$

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \text{ (equation } \mathbf{2}\text{)}$$

The denominator in equation 2 is the total light ($2I_{\perp}$ to take account of both perpendicular planes); *A* therefore provides the most algebraically convenient description of FP because anisotropy components can be summed (polarization values cannot) [4]. A fluorescent molecule with the properties described above would then (with no rotation) have:

$$P = \frac{60 - 20}{60 + 20} = 0.5$$

$$A = \frac{60 - 20}{100} = 0.4$$

If the molecule tumbles so that it becomes randomly re-oriented during the fluorescent lifetime, then both *A* and *P* become 0 (because $I_{\parallel} = I_{\perp}$). With other conditions (viscosity, etc) constant, size determines whether the molecule can re-orient during the fluorescent lifetime [2, 3]. For large molecules, the speed of tumbling is slower than typical fluorescent lifetimes; now a molecule is likely to retain the same orientation throughout the interval between absorbing and emitting a photon. Binding of a fluorescent ligand (D*) to a larger protein (R) increases the effective size of the fluorescent complex and it therefore tumbles more slowly, causing more emitted light to remain polarized with respect to the excitation light (Fig. 1B). This allows FP to be used as a non-perturbing assay of ligand binding (Fig. 1A-C).

[Fig 1 near here]

Inositol 1,4,5-trisphosphate receptors (IP_3R) are intracellular channels, which are expressed in the endoplasmic reticulum of almost, if not all cells [5]. They mediate the initial release of Ca^{2+} from intracellular stores evoked by cell-surface receptors that induce IP_3 formation and contribute to regulating a myriad of cellular processes [5]. The functional channel is a tetramer of the same or different subunits encoded by three different IP_3R genes (IP_3R1-3) and its opening requires binding of both IP_3 and Ca^{2+} [6]. The IP_3-binding core (IBC, residues 224-604 of IP_3R; Fig. 2A) of each subunit is entirely responsible for binding IP_3 [7]. Residues immediately preceding the IBC (residues 1-223, suppressor domain; SD) decrease the IP_3-binding affinity. Thus, the IBC displays substantially higher affinity for IP_3 than the full-length IP_3R or an N-terminal fragment comprising the SD and IBC (residues 1-604; N-terminal; NT) [8] (Fig. 2A). IP_3 binding initiates channel activation by closing the clam-like IBC and displacing the SD [9, 10], which is essential for channel gating [8, 11]. These conformational changes are probably transmitted directly to the channel pore *via* direct contacts between

the IBC and the C-terminal domain (CTD) (Fig. 2A), which is contiguous with the pore [12]. The IBC and NT fragments can be expressed as soluble proteins in bacteria and recognise IP_3 and related ligands with the same specificity as native IP_3R [11, 13]. Here we use IBC and NT fragments of IP_3R1 to demonstrate the utility of FP for analyses of ligand binding to IP_3R [14]. For these analyses we use IP_3 labelled at its 2-position with fluorescein isothiocyanate (FITC-IP_3) because 2-modified analogues of IP_3 bind to the IBC and activate the IP_3R [11]. We initially confirmed the validity of the FP assay by measuring the affinities of FITC-IP_3, IP_3 and adenophostin A (Fig. 2B) to the NT and IBC and the results were comparable to those obtained using conventional radioligand binding assays [14]. Adenophostin A is a metabolically stable, high-affinity agonist of IP_3Rs and residues within the IBC are sufficient to confer the high-affinity binding [13]. Typical results obtained using the FP assay are shown in figure 2. These include an equilibrium saturation curve with FITC-IP_3 and increasing concentrations of the NT (Fig. 2C), and equilibrium competition curves using IP_3 or adenophostin A as competing ligands and a fixed concentration of the NT (Fig. 2D). In a typical equilibrium competition binding assay, a single 96-well plate provides sufficient data to determine the K_D for 3 different ligands (using 15 concentrations of each ligand in duplicates). The non-destructive nature of the assay allows analysis of the effects of temperature on ligand binding. The same 96-well plate can be used repeatedly to measure the K_D of the 3 ligands at 6 different temperatures in less than 3 hours. These analyses can then provide estimates of the enthalpy (ΔH°) and entropy (ΔS°) changes associated with ligand binding (Fig. 2E). Re-using the same plate for repeated measurements reduces the variability due to plate-to-plate differences and it makes efficient use of precious materials. Similar experiments using radioligand binding assays would have been impracticable due to the need of expensive radioligands, time (i.e. would have taken several days to complete) and the fast off-rates at high temperatures.

[Fig 2 near here]

Our FP assay, once optimized, provides a rapid (1-3 hours) and relatively inexpensive way of measuring ligand binding to IP_3R and its fragments in real-time. Since it does not require separation of ligand and protein, it can detect even low-affinity interactions. The assay avoids use of radioactive materials, it is non-destructive and it can resolve Gibbs free energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) changes of ligand binding. The protocol is divided into 5 steps comprising optimization (3.1), preparation of plates for equilibrium saturation (3.2) or competition (3.3) binding assays, anisotropy measurements (3.4) and data analysis (3.5). We describe the methods to use with the NT and FITC-IP_3. Other fragments and fluorescent ligands can be used, but the protocol would need to be modified accordingly.

2. Materials

1. Ca^{2+}-free cytosol-like medium (Ca^{2+}-free CLM): 140mM KCl, 20mM NaCl, 2mM $MgCl_2$, 1mM EGTA and 20mM PIPES, pH 7.0. Use ultrapure water (purified using Milli-Q or similar) and analytical grade reagents. The solution can be stored at 4°C for several months.
2. "FITC-IP_3" (D-2-*O*-(2-(3-(5-fluoresceinyl)thioureido)ethyl)-*myo*-inositol 1,4,5-trisphosphate). FITC-IP_3 was synthesized and purified as described [14] (*see* **Note 1**).
3. Purified N-terminal fragments of IP_3R. N-terminal fragments of IP_3R1 were expressed in *E. coli* and then purified and quantified (*see* **Note 2**) as previously described [1]. We used the N-terminal (NT, residues 1-604 of IP_3R1) and the IP_3-binding core (IBC, residues 224-604 of IP_3R1), but other fragments or mutant versions could be used.
4. Half-area, black, flat-bottomed, polystyrene 96-well microplates.
5. Inositol 1,4,5-trisphosphate hexapotassium salt (IP_3) and other competing ligands (*see* **Note 3**).
6. Bench-top centrifuge for 96-well plates.
7. Multi-channel (8-channel) for using with 96-well plates and tips.
8. Plate-reader suited for FP measurements (eg, PHERAstar, BMG Labtech).

9. Curve-fitting software.

3. Methods

3.1 Optimization of an FP assay for N-terminal fragments of IP_3R (*see* **Note 4**).

3.1.1 Assess whether the fluorescence intensity of the ligand (here FITC-IP_3) changes upon binding to NT (*see* **Note 5**).

1. Prepare 15 dilutions of NT (with final concentrations ranging between 1-300nM) by serially diluting the purified NT sample ("serially diluted NT").
2. Mix each concentration of purified protein with FITC-IP_3 (final concentration, 0.5nM) in Ca^{2+}-free CLM.
3. Load duplicate 50-μl samples of each dilution into individual wells of a 96-well plate.
4. Centrifuge the plate (300x*g*, 2min, 20^{o}C).
5. Incubate for 20min to allow equilibrium to be attained.
6. Measure the intensity of the fluorescence emitted at 520nm with excitation at 485nm using a suitable plate-reader (eg, PHERAstar). Note that no measurements of polarized light are required in this step.

3.1.2 Establish the optimal concentration of FITC-IP_3 to be used in the assay.

1. Prepare serial dilutions of FITC-IP_3 (final concentrations, ~0.1nM to ~5nM) in Ca^{2+}-free CLM.
2. Load duplicate 50-μl samples of each dilution into a 96-well plate.
3. Centrifuge the plate (300x*g,* 2min, 20^{o}C).
4. Measure fluorescence intensity in the parallel ($I_{\parallel}$) and perpendicular planes ($I_{\perp}$) and thereby anisotropy (*A*) (see equation 2) using the PHERAstar plate-reader as described in "Anisotropy measurements".
5. Choose the lowest concentration of FITC-IP_3 (to keep it below its equilibrium dissociation constant, K_D) that allows *A* to be reliably measured. Here we used 0.5nM FITC-IP_3.

3.1.3 Assess whether the protein interferes with the detection of polarized light (*see* **Note 6**).

1. Mix “serially diluted NT” (final concentrations, 1-300nM) with FITC-IP_3 (final concentration, 0.5nM) and saturating IP_3 (final concentration, 10μM) in Ca^{2+}-free CLM.
2. Load duplicate 50-μl samples into a 96-well plate.
3. Mix by shaking the plate gently (30rpm, 20^{o}C) using the orbital shaking mode in the PHERAstar plate-reader (or an orbital shaker).
4. Centrifuge (300x*g,* 2min, 20^{o}C).
5. Incubate for 20min and then measure $I_{\parallel}$ and $I_{\perp}$ (and thereby *A*) using the PHERAstar plate-reader as described in “Anisotropy measurements”.

3.2 Preparation of plates to perform FP equilibrium saturation binding assays

1. Prepare 15 dilutions of the purified NT (with final concentrations ranging between 1-300nM) by serially diluting the purified NT sample (“serially diluted NT”).
2. Load a 96-well plate with duplicate 50-μl samples in Ca^{2+}-free CLM of:
   a. Each dilution of “serially diluted NT” and FITC-IP_3 (final concentration, 0.5nM) to determine A_M (the measured *A*, Fig. 1C) for each NT concentration, and A_{D^*R} (the anisotropy of bound fluorescent ligand, D^*, Fig. 1C) from the well containing the highest concentration of NT;
   b. Each dilution of “serially diluted NT” and FITC-IP_3 (final concentration, 0.5nM), with a saturating concentration of IP_3 (final concentration, 10μM) to determine A_I (*A* in the presence of saturating competing ligand, Fig. 1C) and thus A_{NS} (*A* due to nonspecific binding of D^*, Fig. 1C) at each NT concentration;
   c. Each dilution of “serially diluted NT” alone to measure background fluorescence ($I_{\parallel}$ and $I_{\perp}$) at each NT concentration. If background fluorescence values are significant, they must be subtracted from the equivalent measurements in the presence of FITC-IP_3 before calculating *A*;

d. FITC-IP_3 (final concentration, 0.5nM) to determine A_{D^*} (the anisotropy of free D, Fig. 1C).

Each of these conditions (a-d) should be included in every plate.

3. Mix by shaking plate (30rpm, 15min, 20^{o}C) using the orbital shaking mode in the PHERAstar plate-reader (or an orbital shaker).
4. Wrap plates and samples in foil to minimize exposure to light.
5. Centrifuge plate (300x*g*, 2min, 20^{o}C).
6. Incubate the plate in the dark at the desired temperature (4-37°C) for 20min to allow equilibrium to be attained (*see* **Note 7**).
7. Using the PHERAstar plate-reader, measure $I_{\parallel}$ and $I_{\perp}$ (and thus *A*).

3.3 Preparation of plates to perform FP equilibrium competition binding assays.

1. Prepare 8 dilutions of competing ligand (eg IP_3) with final concentrations ranging between 3nM-150μM) by serially diluting the stock ("serially diluted competing ligand") (*see* **Note 8**).
2. Load a 96-well plate with duplicate 50-μl samples in Ca^{2+}-free CLM containing:
   a. Each dilution of "serially diluted competing ligand", FITC-IP_3 (final concentration, 0.5nM) and NT (final concentration, 80nM) to determine A_M at each concentration of competing ligand, and A_I at a saturating concentration of the competing ligand;
   b. FITC-IP_3 alone (final concentration, 0.5nM) to measure A_{D^*};
   c. FITC-IP_3 (final concentration, 0.5nM) and a saturating concentration of NT (final concentration, 300nM), to determine A_{D^*R} (under these conditions all FITC-IP_3 should be bound) (*see* **Note 9**);
   d. NT (final concentration, 80nM) to measure background $I_{\parallel}$ and $I_{\perp}$.
3. Mix by shaking plate (30rpm, 15min, 20^{o}C) using the orbital shaking mode in the PHERAstar plate-reader (or an orbital shaker).
4. Wrap plates and samples in foil to minimize exposure to light.

5. Centrifuge the plate (300x*g,* 2min, 20^{o}C).
6. Incubate the plate in the dark at the desired temperature (4-37°C) for 20min to allow equilibrium to be attained (*see* **Note 7**).
7. Using the PHERAstar plate-reader, measure $I_{\parallel}$ and $I_{\perp}$ (and thus *A*).

3.4 Anisotropy measurements.

Here we describe the methods to use a PHERAstar fluorescence polarization plate-reader. Similar equipment from other providers is also suitable, but modified procedures will be required.

1. Open the PHERAstar software and select the FP option. Insert the FP 485/520/520 optic module. Set the following options: 3 cycles of measurement, 300 flashes per cycle, 1s positioning delay, and reading direction 11.
2. Use the well containing only 50μl of 0.5nM FITC-IP_3 to perform auto-adjustment of focal height and gain. The 'mP Target' value should be set to 25. The focal height for 50μl of solution in a half-area well should be ~6.3 (*see* **Note 10**),
3. With the above settings take measurements from every well in the plate. The samples are excited with light polarized in the horizontal plane at 485nm, and emitted light at 520nm is collected simultaneously in both the horizontal (parallel, $I_{\parallel}$) and vertical (perpendicular, $I_{\perp}$) planes.
4. For repeated measurements of the same plate at different temperatures, both plate-reader and plate should be re-equilibrated to the desired temperature. After equilibration and before performing measurements, the plate should be incubated for 20min at each temperature to allow reactions to attain equilibrium (*see* **Note 11**).

3.5 Data analysis

Before proceeding with analysis, prepare suitable spreadsheets (eg, Microsoft Excel) to analyse the data directly imported from the plate reader.

1. For each measurement, subtract the background fluorescence $I_{\parallel}$ and $I_{\perp}$ caused by the presence of protein (here NT) alone. Use these corrected values to calculate the measured *A* ($A_{M,}$ see Fig. 1C) using equation 2.
2. Using equation 2, similarly calculate A_I (*A* determined in the presence of a saturating concentration of a competing ligand) from values of $I_{\parallel}$ and $I_{\perp}$ determined for each NT concentration in the presence of a saturating concentration of competing ligand (IP_3). From the calculated A_I and A_{D^*} (*A* for free D^*, here FITC-IP_3), compute A_{NS} (*A* due to non-specific binding, see Fig. 1C) at each NT concentration using:

$$A_{\mathrm{NS}} = (A_{\mathrm{I}} - A_{\mathrm{D}^*})(1 - \mathrm{F_B}) \qquad (\text{equation } \mathbf{3})$$

where F_B (the fraction of bound D^*) is calculated using:

$$\mathrm{F_B} = \frac{A_{\mathrm{M}} - A_{\mathrm{D}^*}}{A_{\mathrm{D}^*\mathrm{R}} - A_{\mathrm{D}^*}} \quad (\text{equation } \mathbf{4})$$

and A_{D^*R} denotes *A* for D^* bound to R.

3. For each determination of A_M and its corresponding A_{NS}, calculate A_S (*A* due to specific binding of FITC-IP_3) using:

$$A_{\mathrm{S}} = A_{\mathrm{M}} - A_{\mathrm{NS}} \quad (\text{equation } \mathbf{5})$$

4. Use the A_S value (calculated from equation 5) directly to plot the saturation binding curve or to calculate the fraction of D^* (fluorescent ligand, here FITC-IP_3) bound to R (receptor, here NT) using:

$$\frac{[\mathrm{D}^*\mathrm{R}]}{[\mathrm{D}^*]_{\mathrm{T}}} = \frac{A_{\mathrm{S}} - A_{\mathrm{D}*}}{A_{\mathrm{D}*\mathrm{R}} - A_{\mathrm{D}*}} \qquad (\text{equation } \mathbf{6})$$

where $[D^*]_T$ denotes the total concentration of D^*.

5. If $[R]_T$ (the total R concentration) is high enough to ensure minimal depletion of the free R concentration [R] after binding D^* (and thereby $[R]_T \sim [R]$), plot directly A_S against $[R]_T$ to obtain a binding curve. If $[R]_T$ is too low to avoid a significant decrease in [R] as it binds to D^*, [R] should be calculated for each condition before plotting the binding curve using:

$$\frac{[D^*]}{[D^*]_T} = \frac{K_D}{K_D + [R]_T} \qquad \text{(equation 7)}$$

where K_D is the equilibrium dissociation constant and $[D^*]$ and $[D^*]_T$ are the free and total concentrations of D^*, respectively. In either case, use any suitable curve-fitting program to fit the results to a Hill equation:

$$\alpha = \frac{\left(\frac{[R]}{K_D}\right)^h}{1+\left(\frac{[R]}{K_D}\right)^h} \quad \text{(equation 8)}$$

where α denotes the fraction of D^* specifically bound, and *h* is the Hill coefficient. From this, determine the K_D^{D*}, that is K_D of the interaction between D^* and R.

6. Once the K_D^{D*} is established, the K_D of any competing ligand (K_D^I) can be computed (*see* **Note 12**). For equilibrium competition binding measurements, calculate A_M and then A_S from each measurement and from these A_S values compute the total concentration (I_{50}) of the inhibitor (I) that displaces 50% of D^* specifically bound. From the I_{50} value and the known $[R]_T$, calculate the free concentration of I (IC_{50}) that displaces 50% of specifically bound D^*. At the I_{50}, 50% of all R are occupied by I, hence:

$$IC_{50} = I_{50} - [R]_T/2 \quad \text{(equation 9)}$$

7. From A_S value measured at the I_{50} ($A_S{}^{50}$), calculate the $[D^*R]$ at the I_{50} ($[D^*R]_{50}$) using:

$$[D^*R]_{50} = [D^*]_T \frac{A_S{}^{50} - A_{D*}}{A_{D*R} - A_{D*}} \qquad \text{(equation 10)}$$

8. The K_D of I for the binding site (K_D^I) is then calculated using:

$$K_D{}^I = \frac{[D^*R]_{50}\ IC_{50}\ K_D{}^{D*}}{([D^*]_T\,[R]_T) + [D^*R]_{50}\,([R]_T - [D^*]_T + [D^*R]_{50} - K_D{}^{D*})} \qquad \text{(equation 11)}$$

9. From experiments performed at different temperatures, the K_D can be calculated from saturation or competition experiments at each chosen temperature. The following equations define the relationships between K_D, ΔG^o (standard Gibbs free energy change), ΔS^o (standard entropy change) and ΔH^o (standard enthalpy change):

$$\Delta G° = RT\ln K_D \qquad \text{(equation } \mathbf{12}\text{)}$$

$$\Delta G° = \Delta H° - \Delta S° \qquad \text{(equation } \mathbf{13}\text{)}$$

where *R* is the universal gas constant and *T* the absolute temperature. If ΔH^o is temperature-independent (ie, the change in heat capacity, $\Delta C = 0$), a van't Hoff plot ($\ln K_D$ *versus* 1/*T*) can be used to obtain $\Delta H°/R$ from the slope. In cases where $\Delta C \neq 0$, the following equation should be used [15]:

$$\Delta G^o_T = \Delta H^o_{T_o} + \Delta C^o_{T_o}(T - T_o) - T[\Delta S^o_{T_o} + \Delta C^o_{T_o} \ln\left(\frac{T}{T_o}\right)] \quad \text{(equation } \mathbf{14}\text{)}$$

where *T* is the actual absolute temperature, and T_o is the reference absolute temperature (here 296 K).

10. Determine ΔH^o, ΔS^o and ΔC^o by least-squares curve-fitting of ΔG^o *versus* T.

4. Notes

1. This protocol is described for FITC-IP_3, but other fluorescent ligands can be used. If using other fluorophores, ensure the excitation and emission wavelengths used are optimal for the chosen fluorophore and that binding to the protein does not affect the fluorescent properties of the ligand (*see* also **Note 5**).
2. Accurate determination of the concentration of functional ligand-binding sites in the purified protein is crucial for all FP experiments. We used conventional radioligand binding assays with ^{3}H-IP_3 to determine the density of binding sites in the purified protein sample [1]. In all analyses, the concentration of purified protein refers to the concentration of functional binding sites.
3. The specific needs of each experiment will determine the need for other ligands (eg, adenophostin A, synthetic analogues of IP_3R, etc [11]).

4. This protocol is described for the NT. For the IBC, use ~10-fold lower concentrations of functional binding sites (because its affinity for IP_3 is ~10-fold higher than the NT). If using other fragments or mutant fragments, determine their K_D for IP_3 by other methods and adjust the concentration of functional binding sites accordingly.
5. For a fluorescent ligand to be suitable for FP analyses, its rate of depolarization, but not its fluorescence intensity, should change when binding to the protein. If the fluorescence intensity of the ligand changes upon binding the protein, try another fluorescent ligand. This might be achieved by either conjugating the fluorophore to a different position or using a different fluorophore.
6. For all FP measurements, it is crucial to determine whether the protein itself interferes with the detection of polarized light. Here measurements are performed in the presence of a large excess of unlabelled ligand in order to prevent binding of FITC-IP_3 to NT. If attenuation of the emitted light is the same in both planes ($I_{\parallel}$ and $I_{\perp}$), it will not affect measurements of *A*. Otherwise a correction will be required.
7. The time required for reactions to attain equilibrium will depend on the protein and ligand, their concentrations and the temperature. The timing must be optimised for each set of conditions. If equilibrium has not been attained before performing measurements for a given set of conditions, optimise the timing by measuring the same plate at regular intervals (eg, every 5min) to follow the increase in *A* overtime. Determine the time at which *A* reaches a plateau, indicating that equilibrium has been attained.
8. The concentrations of the competing ligand used should cover a range of at least 100-fold either side of its K_D for the protein used.
9. The concentration of purified protein used should be optimized to both avoid wasting valuable material and to maximize the dynamic range. Choose the lowest possible protein concentration to ensure that most fluorescent ligand (here FITC-IP_3) is bound

to the protein before addition of competing ligand. We found that protein concentrations that gave *A* greater than 50% of A_{D*R} (Fig. 1C) worked well.

10. Optimally adjust the gain and focal height to achieve maximal sensitivity.
11. High temperatures might affect the stability of the ligand or the protein (e.g., by causing protein damage or ligand degradation). Verify that the effects of increasing temperature are reversible by measuring the plate at the lowest chosen temperature, then equilibrating the plate at the highest temperature, and repeating the measurement after returning the plate to the lowest temperature. The PHERAstar plate reader is equipped with a temperature-control system. If using a plate reader without temperature control, the plate reader should be housed in a temperature-controlled cabinet.
12. In conventional radioligand binding experiments, the Cheng and Prusoff equation can be used to calculate the K_D from the concentration of drug that displaces 50% of specifically bound radioligand (IC_{50}) [16]. In FP experiments, the relationships between total and free concentrations are more complicated, so the K_D^I cannot be calculated using the Cheng-Prusoff equation [17].

5. References

1. Rossi, A.M. and C.W. Taylor, *Analysis of protein-ligand interactions by fluorescence polarization.* Nature Protocols, 2011. **6**: p. 365-387.
2. Weber, G., *Polarization of the fluorescence of macromolecules. I. Theory and experimental method.* Biochemical Journal, 1952. **51**: p. 145-155.
3. Serdyuk, I., N. Zaccia, and J. Zaccai, *Methods in molecular biophysics. Structure, dynamics and function.* 2007: Cambridge University Press. 1120.
4. Hall, M.D., et al., *Fluorescence polarization assays in high-throughput screening and drug discovery: a review.* Methods Appl Fluoresc, 2016. **4**(2): p. 022001.
5. Berridge, M.J., *The inositol trisphosphate/calcium signaling pathway in health and disease.* Physiological Reviews, 2016. **96**: p. 1261-1296.
6. Taylor, C.W. and V. Konieczny, *IP_3 receptors: Take four IP_3 to open.* Science Signaling, 2016. **9**: p. pe1.
7. Yoshikawa, F., et al., *Mutational analysis of the ligand binding site of the inositol 1,4,5-trisphosphate receptor.* Journal of Biological Chemistry, 1996. **271**: p. 18277-18284.
8. Uchida, K., et al., *Critical regions for activation gating of the inositol 1,4,5-trisphosphate receptor.* Journal of Biological Chemistry, 2003. **278**: p. 16551-16560.
9. Bosanac, I., et al., *Structure of the inositol 1,4,5-trisphosphate receptor binding core in complex with its ligand.* Nature, 2002. **420**: p. 696-700.

10. Seo, M.-D., et al., *Structural and functional conservation of key domains in $InsP_3$ and ryanodine receptors.* Nature, 2012. **483**: p. 108-112.
11. Rossi, A.M., et al., *Synthetic partial agonists reveal key steps in IP_3 receptor activation.* Nature Chemical Biology, 2009. **5**: p. 631-639.
12. Fan, G., et al., *Gating machinery of $InsP_3R$ channels revealed by electron cryomicroscopy.* Nature, 2015. **527**: p. 336-341.
13. Rossi, A.M., et al., *Adenophostins: high-affinity agonists of IP_3 receptors.* Current Topics in Membranes, 2010. **66**: p. 209-233.
14. Ding, Z., et al., *Binding of inositol 1,4,5-trisphosphate (IP_3) and adenophostin A to the N-terminal region of the IP_3 receptor: thermodynamic analysis using fluorescence polarization with a novel IP_3 receptor ligand.* Molecular Pharmacology, 2010. **77**: p. 995-1004.
15. Wittmann, H.J., R. Seifert, and A. Strasser, *Contribution of binding enthalpy and entropy to affinity of antagonist and agonist binding at human and guinea pig histamine H_1-receptor.* Molecular Pharmacology, 2009. **76**: p. 25-37.
16. Cheng, Y.-C. and W.H. Prusoff, *Relationship between the inhibition constant (K_I) and the concentration of inhibitor causing 50 per cent inhibition (IC_{50}) of an enzymatic reaction.* Biochemical Pharmacology, 1973. **22**: p. 3099-3108.
17. Nikolovska-Coleska, Z., et al., *Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization.* Analytical Biochemistry, 2004. **332**(2): p. 261-273.

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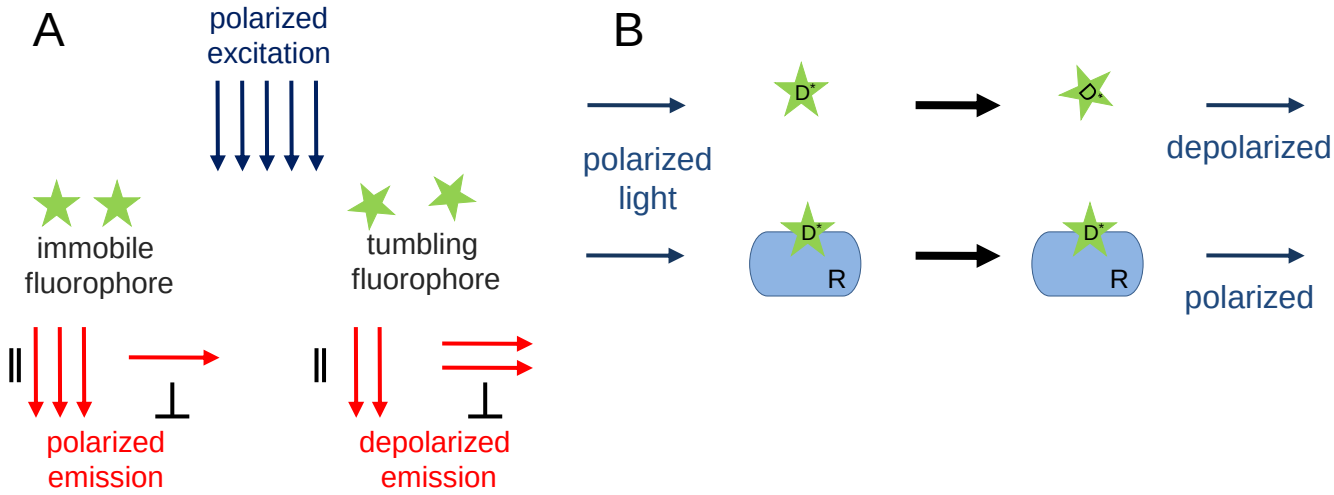
Figure 1. Use of fluorescence polarization to measure ligand binding. (*A*) When an immobile fluorophore is excited with plane-polarized light, 60% of the emitted light remains polarized with respect to the excitation source (i.e. parallel). If the molecule rotates during its fluorescence lifetime, then less than 60% of the emitted light will remain polarized in the parallel plane and more light will be collected in each of the perpendicular planes [3]. FP measures the emission by a fluorophore in two planes (horizontal and vertical) after excitation with polarized light in one of these planes. As described in equation 2, anisotropy (*A*) is defined by the relationships between the intensities of the light detected in the parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) planes with respect to the excitation light. For an immobile fluorescent molecule: $A = (60 - 20)/(60 + 40) = 0.4$. [3]. If the molecule rotates and becomes randomly re-oriented, $I_{\parallel} = I_{\perp}$ and $A = 0$. For a mixture of fluorescent molecules*:*

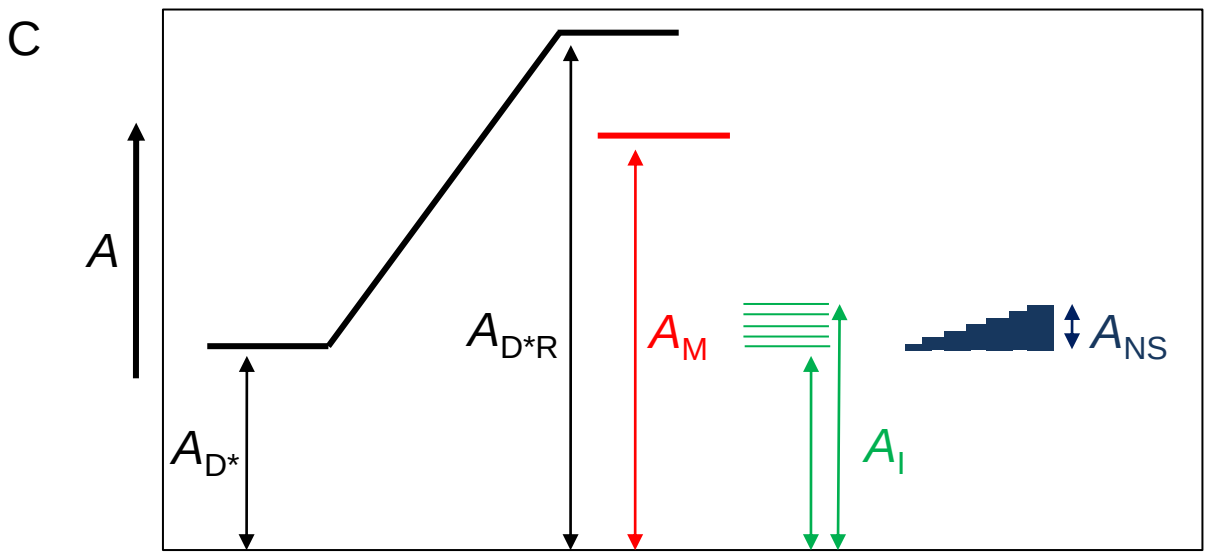
$$A = \sum_{i=1}^{n} f_i A_i$$

where A_i is the anisotropy of each molecule and f_i denotes its fractional fluorescence intensity. As the fraction of reoriented molecules increases, *A* decreases linearly from a 0.4 (its maximum value) to 0. (*B*) A small fluorescent ligand (D^*) free in solution rotates rapidly due to its small molecular volume. If $D^*$is excited with plane-polarized light, it will emit depolarized light and thus will have a low *A* (top). If D^* binds to a large protein (R), its molecular volume will increase and it will therefore rotate less (i.e. the speed of rotation is inversely related to molecular volume [3]). Thus, more of the emitted light will remain polarized with respect to the excitation light, causing *A* to increase (bottom). (*C*) The maximal dynamic range of an FP assay is given by the anisotropy (*A*) of the free D^* (A_{D^*}) and bound D^* (A_{DR^*}). A_M, the measured *A* for each experimental condition, includes *A* from free D^*, and D^* specifically bound to R and non-specifically bound to other components. A_I (for each experimental condition), is the *A* measured in the presence of a saturating concentration of a competing ligand (I) which causes displacement of all D^* specifically bound. A_{NS} (defined as *A* due to non-specific binding) is calculated from A_I using equations

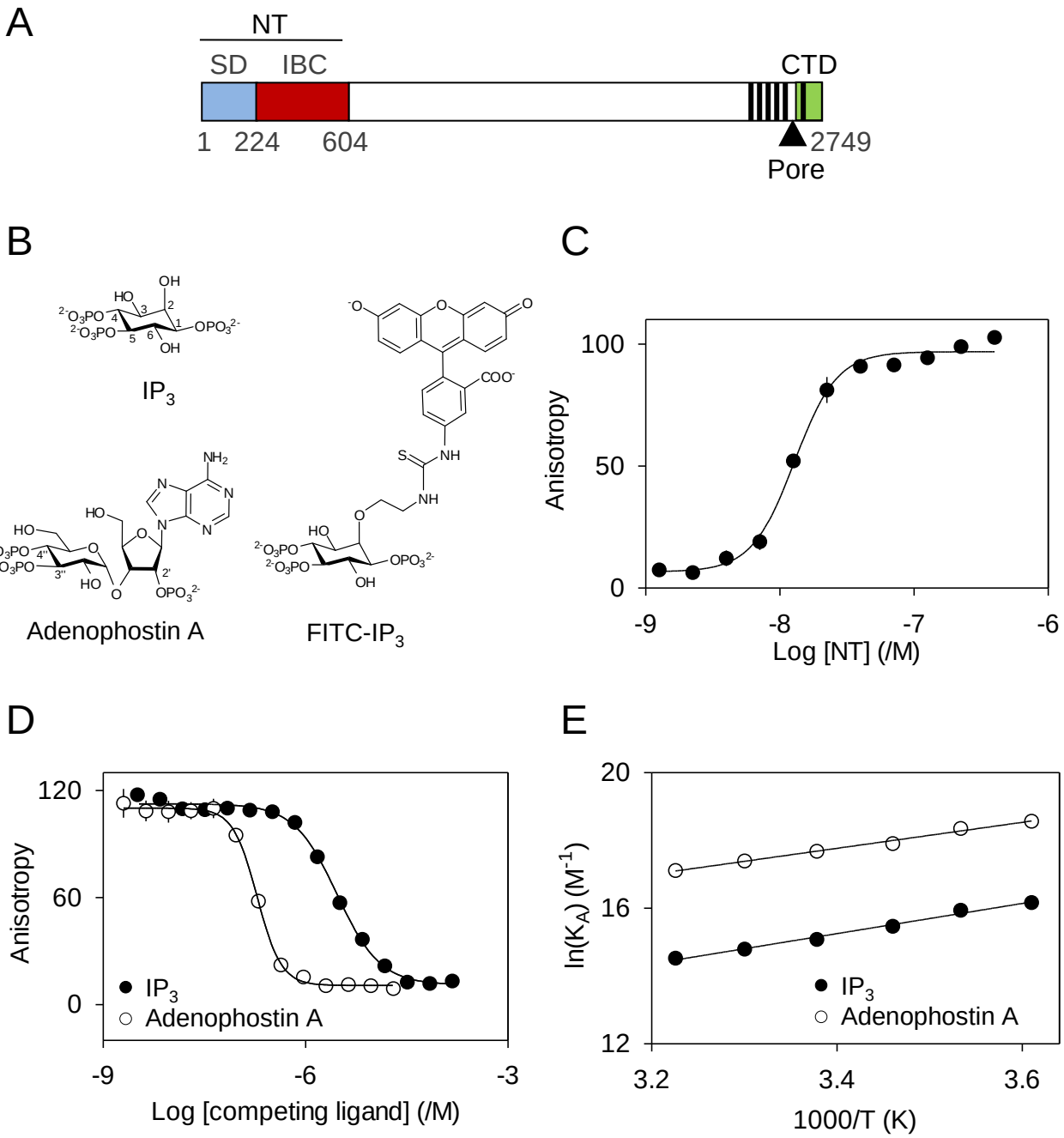
2-4 and A_S (*A* due to specific binding) using equation 5. Reproduced, with modifications from [1].

Figure 2. FP measurements of equilibrium ligand binding to N-terminal fragments of IP_3R (*A*) Cartoon of a single subunit of IP_3R1 showing its key domains: the NT (residues 1-604) which comprises the IBC and SD, 6 transmembrane domains (black lines) and the C-terminal domain (CTD). (*B*) Chemical structures of IP_3, FITC-IP_3 and adenophostin A. (*C*) Anisotropy measurements from a typical FP saturation binding assay using FITC-IP_3 (0.5 nM) and the indicated concentrations of purified NT performed in Ca^{2+}-free CLM at 4°. (*D*) Typical results from a FP competition binding assay using FITC-IP_3 (0.5 nM), NT (80nM) and the indicated concentrations of competing ligands in Ca^{2+}-free CLM at 4°. (*E*) Typical van't Hoff plots for IP_3 and adenophostin A binding to the NT. Results (*C-E*) are means ± S.E.M., *n* = 3. Original data reproduced from [14] with permission from The American Society for Pharmacology and Experimental Therapeutics.





Rossi and Taylor, Figure 1



Rossi and Taylor, Figure 2