Fluorescence Anisotropy/Polarisation: Theory, Method, and Data Analysis

Contents:

Fluorescence Anisotropy/Polarisation

1. Theory

1.1 Introduction to fluorescence anisotropy

Fluorescence Anisotropy is commonly used in biophysical applications of fluorescence. Anisotropy measurements provide information on the size and/or shape of biological molecules and is used frequently to quantitate changes in these parameters. Fluorescence anisotropy have been used to study protein-protein associations, protein-ligand interactions, protein-nucleic acid binding, folding-unfolding of biological molecules and immunoassays. In principle, any biological molecule that has unique intrinsic fluorescence or can be labelled with a unique fluorophore, can be used in fluorescence anisotropy studies.

Anisotropy measurements are based on the principle of selective excitation of fluorophores by polarised light. If the light used to excite a fluorophore is linearly polarised, absorption will be most probable for those fluorophore molecules that happens to lie with their excitation transition moment (dipole) parallel to the plane of polarisation. The excitation transition dipole has a defined orientation with respect to the molecular axis of the fluorophore. The fluorescence emission also occurs with the light polarised along the emitting transition dipole. The relative angle between these two transition dipoles (γ) determines the maximum measured anisotropy (r_0). The term r_0 is used to refer to the maximum anisotropy observed in the absence of other depolarising effects such as rotational diffusion or energy transfer.

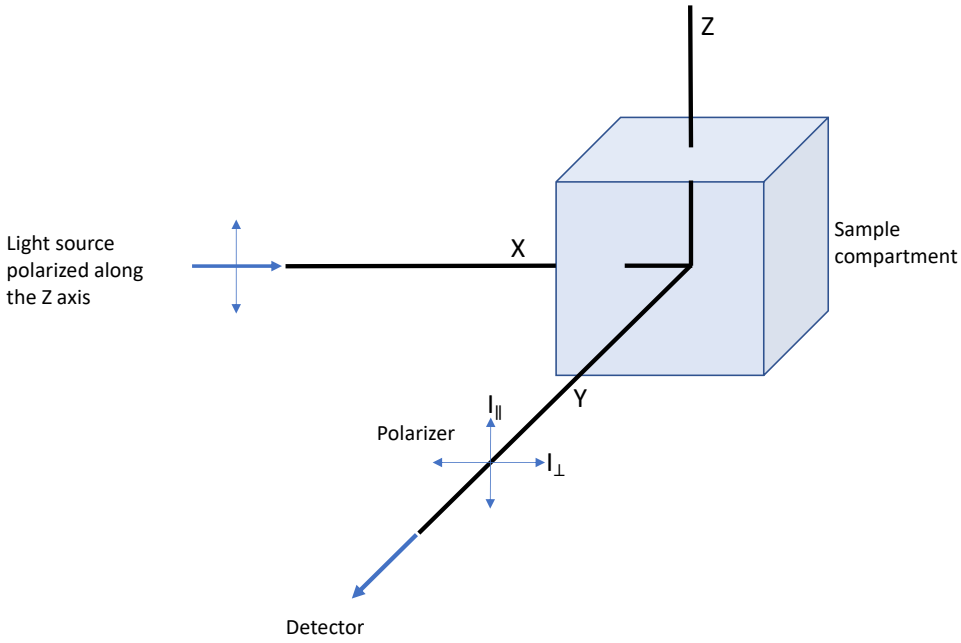

Fig 1: Examples of fluorescence anisotropy experimental set up: The fluorescent moity in the sample compartment is excited along the X axis with linearly polarized light with the electric vector along the Z axis. The emission is detected along the Y axis at right angle to the excitation light, with the polarizer set parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the electric vector of the excitation light (Z-axis). Other experimental set-ups are in use.

The classic L-shaped experimental set up for measuring fluorescence anisotropy is illustrated in Fig 1.

Fluorescence anisotropy (r) is frequently expressed as polarisation (P). Both are expressions of the same phenomenon, both are dimensionless (no units) and both are independent of the total emission intensity. The following equations are used to describe them:

Eq 1.1 $$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}}$$

Eq 1.2 $$P = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + GI_{\perp}}$$

Where: $I_{\parallel}$ is the emission when the detector polarizer is set parallel to Z-axis; $I_{\perp}$ is the emission when the detector polarizer is set perpendicular to Z-axis; and, G is a correction factor (some time called the grating factor) for background scattering intensities introduced by the monochromator grating and is different for individual instruments and experimental setups.

The mathematical relationship between r and P is:

Eq 1.3 $$r = \frac{2P}{(3 - P)}$$ Eq 1.4 $$P = \frac{3r}{(2 + r)}$$

However, it is preferable to use anisotropy (r) because the polarization (P) denominator, $I_{\parallel} + I_{\perp}$, is not proportional to the total amount of emitted light[1].

1.2 The magnitude of fluorescence anisotropy

Several phenomena can decrease the measured anisotropy. The most common cause is rotational diffusion. Such diffusion occurs during the lifetime of the fluorophore excited state resulting in the displacement of the emission transition dipole. In aquas solutions small fluorophores such as fluorescein, rotate extensively in 50 to 100 picoseconds (ps; 10^{-12} seconds) timescale. Hence, during the excited state lifetime of commonly used fluorophores (fluoresceine average lifetime ≈ 4 ns) of 0.5 to 10 nanoseconds (ns; 10^{-9} seconds), the molecule can rotate many times and the orientation of the emission transition dipole becomes randomised. For this reason, free fluorophores in aquas solutions display anisotropy near zero (free fluorescein r = 0.0236). The effect of rotational diffusion can decrease if the small fluorophore is bound to a macromolecule such as Human Serum Albumin (HSA; 67 kDa). The rotational correlation time for HSA is 50 ns. Suppose fluorescein is covalently attached to HSA, then the expected anisotropy can be calculated using the Perrin equation (equation 1.5):

Eq 1.5 $$r = \frac{r_0}{1 + (\tau/\tau_c)}$$

Where, r_0 is the maximum anisotropy in the absence of rotation, τ is the fluorescein excited state lifetime, and τ_c is the HSA rotational correlation time. Assuming r_0 is 0.4 (see next paragraph) then the anisotropy of fluorescein bound HSA is 0.37. Smaller globular proteins would be expected to yield lower anisotropies and large proteins bound to longer lifetime fluorophores would also yield lower anisotropies. The essential point is that the rotational correlation times of most proteins are comparable to the fluorescence lifetime of typical fluorophores. For this reasons, measurements of fluorescence anisotropy are widely used to study the interactions of biological macromolecules.

To understand the maximum experimentally derived anisotropy it is best to utilise a thought experiment (Fig 2). We assume a simple situation in which the fluorophore excitation and emission transition dipoles are parallel (the angle between the two dipoles, $\gamma = 0^{\circ}$). We subject the sample compartment to a strong magnetic field which orient the fluorophore molecules with their transition dipoles parallel to the plane of polarisation of the excitation light (Z axis, Fig 2a). We flash freeze the sample at very low temperature (-137˚C) to form vitreous ice to prevent any molecular motions.

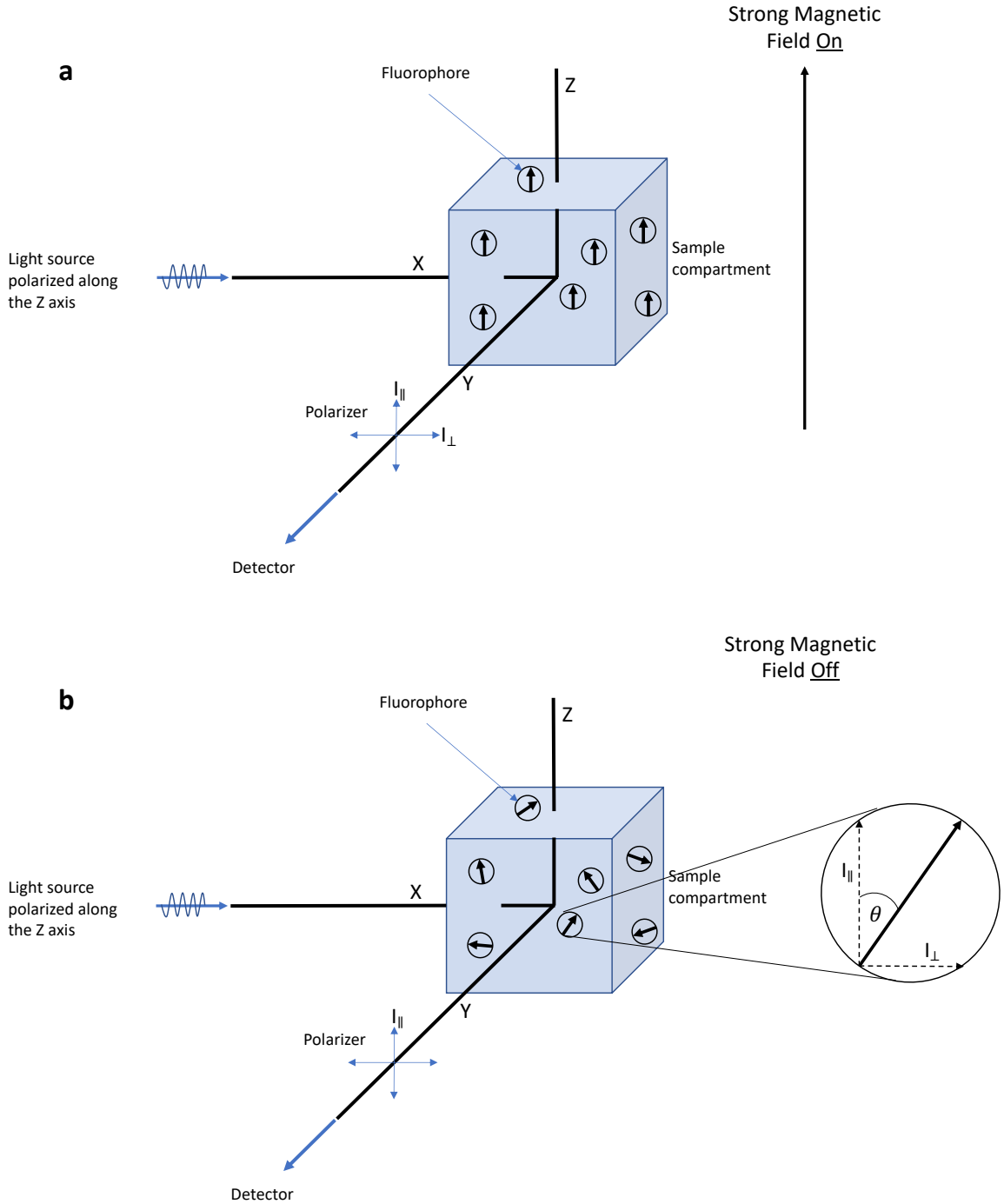

Fig 2: a) fluorophores are oriented by a magnetic field with their transition dipoles parallel to the polarised excitation light (Z axis) before excitation; **b**) fluorophores with randomly oriented transition dipoles in the absence of magnetic field. Insert – the resolution of an emission dipole moment vector into parallel and perpendicular components.

We then irradiate the sample with linearly polarised light. Since all the fluorophores are with their excitation and emission dipoles parallel to the plane of the polarisation of the excitation light and no molecular motion takes place during the exited state lifetime, there is no emission in the perpendicular plane ($I_\perp = 0$). Substituting this information in equation 1.1 we obtain anisotropy value equal to 1.

We next turn off the magnetic field and allow the fluorophore solution to equilibrate before we flash freeze to form vitreous ice (Fig 2b). This results in the fluorophore population adopting random distribution with respect to their transition dipoles but prevent any molecular motions (rotational diffusion) before we irradiate the sample with linearly polarised light. We note that the dipole moments are vector quantities and as such can be resolved to a parallel and perpendicular component (Fig 2b insert). Fluorophores that lie at an angle, θ, to the Z axis will absorb and emit (in all directions) polarised light in the parallel and perpendicular planes. The mathematical solution for the emitted light under such conditions require a statistical mechanics approach (see references 1 & 2). For the simplified condition described above where the excitation and emission dipoles are parallel ($\gamma = 0^\circ$) the maximum anisotropy, r_0, is 0.4. When $\gamma \neq 0^\circ$, then r_0 will vary between 0.2 and 0.4 when there is no molecular rotation[2]. Thus, experimentally, the anisotropy measured should not be greater than 0.4 ($P = 0.5$).

Scattered light can interfere with anisotropy measurements. If the measured anisotropy is greater than 0.4 and the parallel and perpendicular intensities are well within the tolerance range of the detector, one can confidently infer the presence of scattered light in addition to fluorescence.

As mentioned above, in the absence of rotational diffusion or energy transfer between the fluorophores, the maximum anisotropy, r_0, is 0.4 when the excitation and emission dipoles are colinear ($\gamma = 0^\circ$). When $\gamma = 54.7^\circ$ the anisotropy value is zero. When γ exceeds 54.7° the anisotropy becomes negative. The maximum negative value for the measured anisotropy is -0.2 when $\gamma = 90^\circ$. In practice, it is rare to come across experimentally derived negative anisotropy. This is because the lowest electronic singlet state is generally responsible for the observed fluorescence and this state is also responsible for the long-wavelength absorption band. Absorption and emission involving the same electronic transition have nearly colinear dipole moments. Larger γ values are obtained upon excitation into higher electronic states, which are generally not the states responsible for fluorescence emission (the fluorophores relax very rapidly to the lowest excited singlet state). For example, the lowest electronic transition of tyrosine (260 to 290 nm) is due to a transition moment oriented across the phenol ring (^1L_b) that give rise to positive anisotropy. Excitation below 240 nm (higher energy) results in negative anisotropy due to a transition moment (^1L_a) nearly perpendicular to ^1L_b. Experimentally derived negative anisotropy is most commonly observed when the emission intensities exceed the detector tolerance.

1.3 Anisotropy and size of macromolecules.

As discussed in section 1.2 the anisotropy is dependent on the rotational correlation time of the macromolecule (small proteins rotate faster than larger ones) and this will be affected by the viscosity, size and shape of the rotating molecule and the temperature. From the Perrin equation (equation 1.5) the anisotropy is also depended on the average fluorescence lifetime of the fluorophore which is sensitive to the pH. Experimentally, we maintain constant temperature and constant viscosity and thrive to operate at the optimum pH for a particular fluorophore. Assuming globular macromolecules, one can calculate the theoretical dependency of the anisotropy on the molecular weights and the fluorescence lifetimes, using the Perrin equation. An example of that is shown in Fig 3:

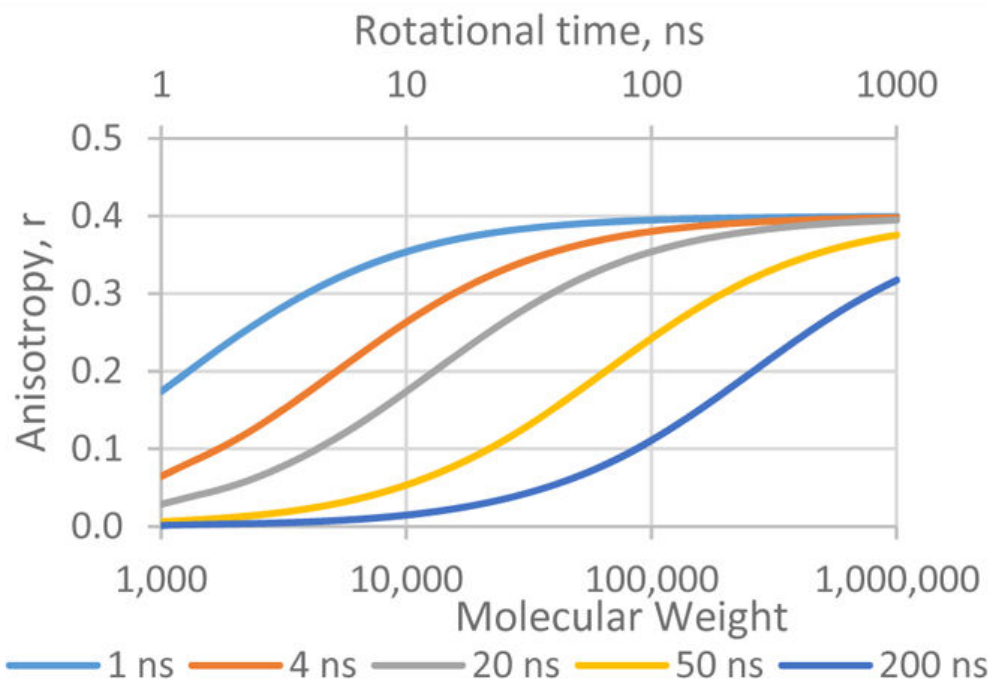

Fig 3: Theoretical anisotropy plotted against log scale MW (bottom axis) and rotational correlation time (top axis) as a function of fluorescence lifetimes. Taken from Zhang *et al.* (2015)[3].

It is abundantly clear from the theoretical plots in Fig 3 that the relationship between anisotropy and the molecular weight is not linear. We assume a hypothetical short peptide of 1 kDa MW labelled with a fluorophore with $\tau = 4$ ns. This peptide can bind 2 protomers of a globular protein of 50 kDa Mw. Then, for the free peptide $r = 0.080$; the binding of the first protomer will result in an anisotropy increase to $r = 0.371$; the binding of the second protomer will result in an increase from $r \approx 0.371$ to $r \approx 0.384$, an increase of only 0.013 anisotropy (compared to 0.291 for binding the first protomer). In this simplified treatment we ignore the complex rotational correlation time for two protomers bound by a short peptide and we assume an overall globular structure for the ternary complex. Thus, quantitative analysis of anisotropy binding data is more suitable for one-to-one binding model.

1.4 The G factor.

In many spectrofluorometers capable of measuring fluorescence anisotropy, monochromators are used for selecting the excitation and emission light waves. These monochromators use diffraction gratings to disperse the polychromatic light source into various wavelengths. The transmission efficiency for a grating monochromator depends on the polarisation of the light. Hence, the polarisation characteristics of monochromators have important consequences in the measurements of fluorescence anisotropy. Such measurements must be corrected for the varying efficiencies of each optical component. This correction is expressed as the G factor.

Stray light is defined as any light that passes through the monochromator besides the desired wavelength. The stray light level of the monochromator is a critical parameter for fluorescence measurements. Stray light at longer wavelengths than the selected one can be passed by the excitation monochromator and can easily be as intense as the fluorescence itself. This is important because it is not unusual for the fluorescence signal to be 1000-fold less intense than the exciting

light. For this reason, double-grating monochromators are frequently used, especially for excitation. However, double-grating monochromators are less efficient, and sensitivity must therefore be sacrificed. It is important to recognise that scattered light is highly polarised. This means that scattered light will contribute to the parallel but not to the perpendicular intensity. Therefore, stray scattered light can easily invalidate measurements of fluorescence anisotropy. Another source of unwanted light from diffraction grating is first, second or higher order diffraction processes which frequently overlap resulting in unwanted wavelengths both from the excitation and emission monochromators.

The G factor is calculated using the following equation:

Eq 1.6 $$G = {I_{HV}}/{I_{vv}}$$

Where: I_{HV} is the intensity measured when the excitation polariser is set perpendicular to the Z-axis, and the emission polariser is set parallel to the Z-axis; I_{VV} is the intensity measured when the excitation polariser is set to parallel, and the emission polariser is set to parallel. G factor calculations require spectrofluorometers where the polarisers can be controlled independently. In modern spectrofluorometers the G factor is measured automatically. In some plate readers that use monochromators this is not possible.

The polarisation-depended transmission properties of monochromators can distort the emission intensities. In some cases, a 10% contribution of scattered light to the emission can result in almost twofold error in the measured anisotropy. For this reason, polarisation anisotropy measurements are frequently performed using excitation and emission filters rather than monochromators. Optical filters are used to compensate for the less-than-ideal behaviour of monochromators and to remove unwanted wavelengths from the excitation and emission beams. A large range of filters are available, such as coloured glass or more commonly, thin film filters. Some colour filters are called long-pass filters that transmit all wavelengths above a particular wavelength. Other filters transmit a selected range of wavelengths (interference filters). The use of filters generally removes stray light and increases the sensitivity because the band-pass of the observation is increased and the attenuation due to the monochromator is removed. The signal level can often be 50-fold higher when observed through filters rather than a monochromator.

2. Methods

This section describes methods for measuring fluorescence anisotropy using plate readers (e.g., PHERAstar FS from BMG Labtech). The methods described can easily be translated to spectrofluorometers.

2.1 Selection of fluorophores.

For one-to-one binding event it is necessary to decide which specie we wish to label with a fluorophore (unless of course we are studying the folding/unfolding of a single macromolecule or using intrinsic fluorophore, e.g., Tryptophan). Because we are monitoring changes in rotational correlation time, we wish to observe a significant magnitude of change in this parameter. This is best achieved by attaching the fluorophore to the smaller of the two interacting species. The binding of the larger specie will result in a significant increase in the rotational correlation time and thus a significant increase in the anisotropy magnitude.

The fluorescence lifetime (τ) of the dye is a key parameter that needs to be considered in fluorophore selection. For best sensitivity, the fluorescence lifetime has to match the rotational correlation time (τ_c) of the target. As mentioned in section 1.2, τ_c lies within a range of several tens of picoseconds for small molecules, such as free fluorescence dyes in solution, to tens of nanoseconds for large macromolecules. The effect of rotation on the *r* values is strongest when the fluorophore emits on the same time scale as the rotation. If the fluorescence lifetime is much shorter than the rotational correlation time ($\tau \ll \tau_c$) then the fluorescence emission is over before molecular rotation is complete, making the determination of anisotropy problematic. If the fluorescence lifetime is much longer than the rotational correlation time ($\tau \gg \tau_c$) then the fluorescent anisotropy will become completely depolarised due to extensive rotation of the target, showing low anisotropy. For small targets, such as spherical proteins of less than 10 kDa, fluorescence dyes with short lifetime (~ 1 ns), such as Cyanine dyes, are preferable. For larger targets (10 to 50 kDa) fluorescence dyes with longer lifetimes (~ 4 ns), such as fluorescein and Bodipy dyes, are preferable. For targets larger than 50 kDa dyes with at least 10 ns lifetime are required (Dansyls dyes).

The brightness of a fluorophore (*B*) is the numbers of photons per second observed for a single fluorophore molecule under a given set of optical conditions. Unlike the quantum yield (*Q*), the brightness is not a molecular property of the fluorophore, but depends on the intensity of the excitation light, the light collection efficiency of the instrument (optics), and the counting efficiency of the detector. The fluorescence anisotropy value (*r*) of a mixture of bound and unbound species is supposed to be directly related to the corresponding fractions of the bound and unbound. This is valid for fluorophores when the brightness in the bound and unbound states is the same. Traditional probes with a rigid geometry in the excited state, such as fluorescein, porphyrines, and some rhodamines, do not significantly change brightness upon interaction with proteins. Fluorophores with flexible excited state geometry, such as the cyanine dyes, exhibit major changes in brightness in response to changes in the microenvironment, thus leading to potential error in calculating the bound fraction and subsequently the equilibrium dissociation constant (K_d). It is imperative that, when performing fluorescence anisotropy experiments, the total emission intensity ($I_{\parallel} + 2I_{\perp}$) should be calculated to determine if there is any change in the brightness of the fluorophore due to binding. Most modern instruments employed for measuring fluorescence anisotropy automatically calculate the total emission intensity. In cases where change in the brightness is observed, the bound fraction is corrected using a simple mathematical approach (see section 3.3)

Ideally, the fluorophore should be tightly associated with the target in the bound state. However, this is difficult to achieve synthetically. Consider a short peptide or DNA labelled at one

of the ends with a fluorophore. Methods for incorporating the fluorophore, such as N-hydroxysuccinimide (NHS) or maleimide linkage, requires linkers that are often made from aliphatic chains. These chains have freely rotating bonds that leads to higher local mobility of the fluorophore ('propeller effect') and therefore, faster depolarisation (lower *r* values). If the binding of the labelled peptide (or DNA) to a protein has little or no effect on the fluorophore microenvironment, the change in the observed anisotropy might be quite small. Sometime this is unavoidable but should be considered when designing the labelled specie. A significantly lower than expected *r* value for the fully bound state is usually an indication of such a 'propeller effect'.

2.2 How much labelled specie.

When preforming fluorescence anisotropy, it is important to obtain good signal to noise while at the same time using the lowest amount of the fluorophore-labelled specie (see section 3.2 for explanation). Since the detection of emission varies from instrument to instrument, it is best to determine the working concentration of the fluorophore-labelled specie empirically.

One must generate a serial dilution of the fluorophore-labelled specie in the assay buffer and measure the anisotropy which should be independent of the concentration. For labelled short peptides or DNA and using a plate reader (PHERAstar FS), we routinely generate a serial dilution from 1 to 100 nM. In addition, it is necessary to calibrate the instrument against a known standard. We routinely use free fluorescein (Fluorescein sodium salt; Merck catalogue number 30181) in 50 mM Tris-HCl pH 8.0 at 5 nM concentration for anisotropy measurements of fluorescein or Alexa-Fluor 488.

To generate a serial dilution, we use the following method. Prepare 220 µl of 100 nM concentration of the fluorescently labelled specie in the assay buffer, in an Eppendorf tube (Fig 4, tube number 1). If the degree of labelling is known (the number of fluorescence dye molecules covalently attached to the labelled specie) calculate the concentration using the extinction coefficient (ε) of the fluorophore. With commercially acquired peptides and DNAs this is straight forward as generally only one fluorophore dye molecule is attached. With in-house labelling the degree of labelling has to be determined by mass spectroscopy or other means. One has to remember that the fluorophore contributes to the absorption in the far-UV range, so it is not possible to determine the concentration from the absorbance at 280 nm or 260 nm. Prepare 7 more Eppendorf tubes with 110 µl assay buffer in each. Using the same pipette and pipette tip transfer 110 µl from tube 1 to tube 2 and mix well by aspirating the solution up and down several times (7 to 10 times). Transfer 110 µl from tube 2 to tube 3 in the same way with the same pipette and pipette tip and repeat up to tube 8 which will end up with 220 µl in it. This will generate a 2-fold dilution series from 100 nM to 0.781 nM. With a fresh tip transfer 100 µl from tube 8 to a well in a 96 well plate (96 well, half area, black plate, flat bottom, NBS; Corning). Using the same tip, continue to transfer 100 µl from each tube into the next well going up the dilution series. Transfer 100 µl 5 nM fluorescein in 50 mM Tris-HCl pH 8.0 into a well (reference).

In the PHERAstar plate reader set the gain and the Z-height (the focal depth in the well) using the fluorescein reference well. Set the milli-polarisation (mP) to 35 ($r = 0.0236$) and the intensity to 20%. The gain values should be somewhere between 300 to 1800 for channels A and B. Using these values, measure the anisotropies of the dilution series. Next, set the gain and the Z-height for the high labelled-specie concentration and the intensity to 95%. Measure the anisotropies. The precise high concentration to use as reference is determined empirically and is governed by the brightness of the fluorophore attached. The guiding principle is that the fluorescein reference should read close to 35 mP.

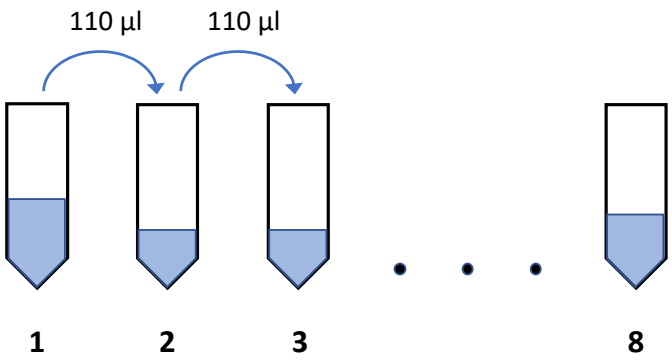

Fig 4: Illustrating a general method for the generation of a dilution series of the fluorescently labelled specie.

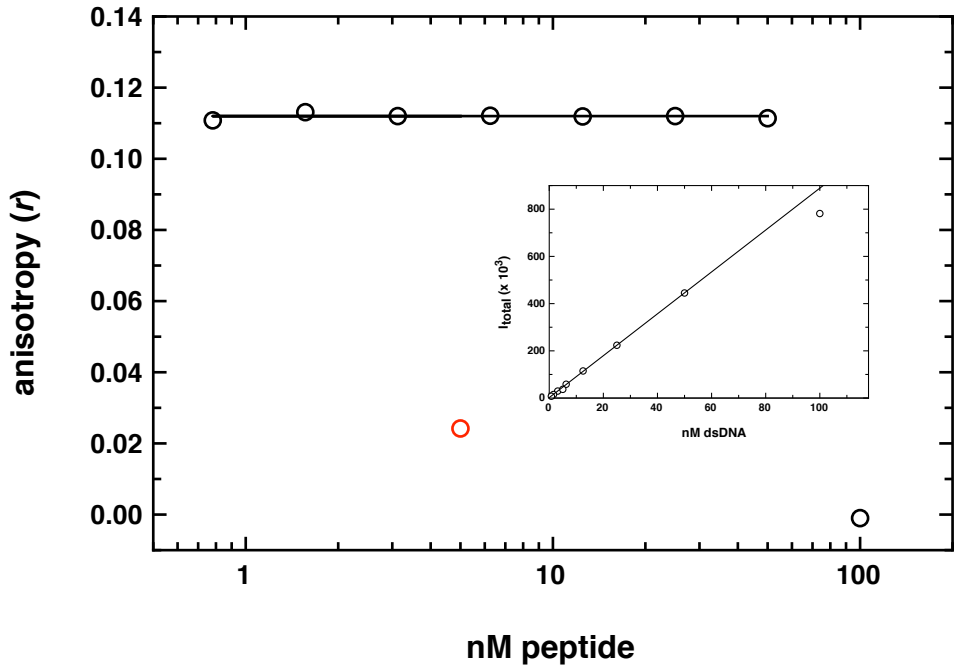

Fig 5: Anisotropy of serial dilution of 109 amino acids long peptide labelled with Alexa Fluor 488. The horizontal line through the data points from 0.781 nM to 50 nM represent the anisotropy of this construct which is independent of the concentration and is equal to 0.112 (160 mP). The red open circle is the anisotropy of 5 nM fluorescein (r = 0.024; mP = 36). The insert shows the linear increase in the total intensity as a function of the construct concentration.

A particularly bright fluorophore is shown in Fig 5 where the anisotropy of the Alexa Fluor 488 labelled peptide does not vary as the concentration increases from less than 1 nM up to 50 nM. The total intensity increase shown in the insert demonstrate that at 100 nM concentration the intensity increase is no longer linear due to saturation of the detectors. Such a plot allows the determination of the anisotropy of the fluorescently labelled unbound specie as well as the concentration necessary to preform anisotropy experiments. In this case, 1 to 2 nM Alexa Fluor 488 labelled peptide is sufficient.

2.3 Experiment setup.

Having established the working concentration of the fluorophore labelled specie one can now proceed with the actual experiment. It is important first to establish the range of concentrations of the non-fluorescent specie (the binding macromolecule). If one has an idea of what the K_d is, then it is important to have at least 5 concentration points below the K_d. Concentration points above the K_d should extend to at least ten times the K_d, a point at which 91% of the fluorophore labelled specie is bound to the macromolecule. If the K_d is unknown, then it is necessary to determine the range by preforming experiments at several ranges covering at least 4 to 5 orders of magnitude of concentrations.

We use the following method. Prepare 300 µl of high binding macromolecule concentration in the assay buffer, in an Eppendorf tube (Fig 6, tube number 1). Prepare 16 more Eppendorf tubes with 100 µl assay buffer in each. Using the same pipette and pipette tip transfer 200 µl from tube 1 to tube 2 and mix well by aspirating the solution up and down several times (7 to 10 times). Transfer 200 µl from tube 2 to tube 3 in the same way with the same pipette and pipette tip and repeat up to tube 16 which will end up with 300 µl in it. Tube 17 contains the assay buffer only. This will generate a ⅔-fold dilution series. With a fresh tip transfer 90 µl from tube 17 to a well in a 96 well plate (96 well, half area, black plate, flat bottom, NBS; Corning). Using the same tip continue to transfer 90 µl from each tube into the next well going up the dilution series.

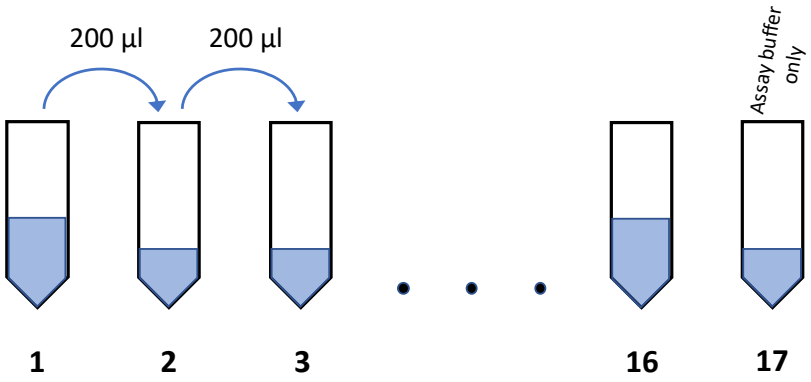

Fig 6: Illustrating a general method for the generation of a dilution series of the binding macromolecule.

To each well add 10 µl of 10 times the working concentration of the fluorophore labelled specie (e.g., 10 µl of 80 nM fluorophore labelled specie will give 8 nM final concentration) using a fresh tip each time. Use the PHERAstar plate reader settings to shake the plate and allow enough time (determined experimentally) to equilibrate at the set temperature. Set the gain and the Z-height using the well containing tube 17 sample (the unbound labelled specie). Set the appropriate mP (160 mP; e.g., Fig 5) and the intensity to 30%. The gain values should be somewhere between 300 to 1800 for channels A and B. Using these values, measure the anisotropies of the dilution series.

Plot the preliminary data on a semi-Log plot (concentration of binding macromolecule in Log scale. See Fig 7). The data should have a sigmoid shape covering 4 to 5 orders of magnitude (Fig 7; 4 to 1850 nM) to ensure complete coverage. The insert in Fig 7 demonstrate that the total intensity is not significantly affected by the binding of increasing concentrations of the macromolecule to the labelled peptide. This indicates that brightness of the fluorophore is not affected by the binding of the macromolecule. The maximum anisotropy value of r = 0.165 (Fig 7) where almost all labelled peptide is bound to the obligate heterodimer. Utilising the Perrin equation (Eq 1.5) and using $r_0 = 0.4$ and $\tau \cong 4$ ns, we calculate the rotational correlation time, τ_c, to be about

2.7 ns. This is significantly lower for what we expect from a 28 kDa heterodimer bound to a 60 amino acids long peptide (Fig 3). A 40 kDa globular protein would be expected to have a rotational correlation time of around 15 ns and a maximum anisotropy of around 0.3. This suggests that we are observing a 'propeller effect' where the Alexa Fluor 488 dye is attached to the peptide (maleimide linkage) through freely rotating bonds and is not directly interacting with the heterodimer.

In addition to this preliminary experiment, one should also check the anisotropy of a sample of the highest concentration of the macromolecule in the absence of the fluorophore labelled specie as well as a sample of the assay buffer in the absence of macromolecule and fluorophore labelled specie. This is done to ascertain that no scattering take place. As mentioned in section 1.4, scattered light is nearly completely polarised and therefore would easily invalidate measurements of

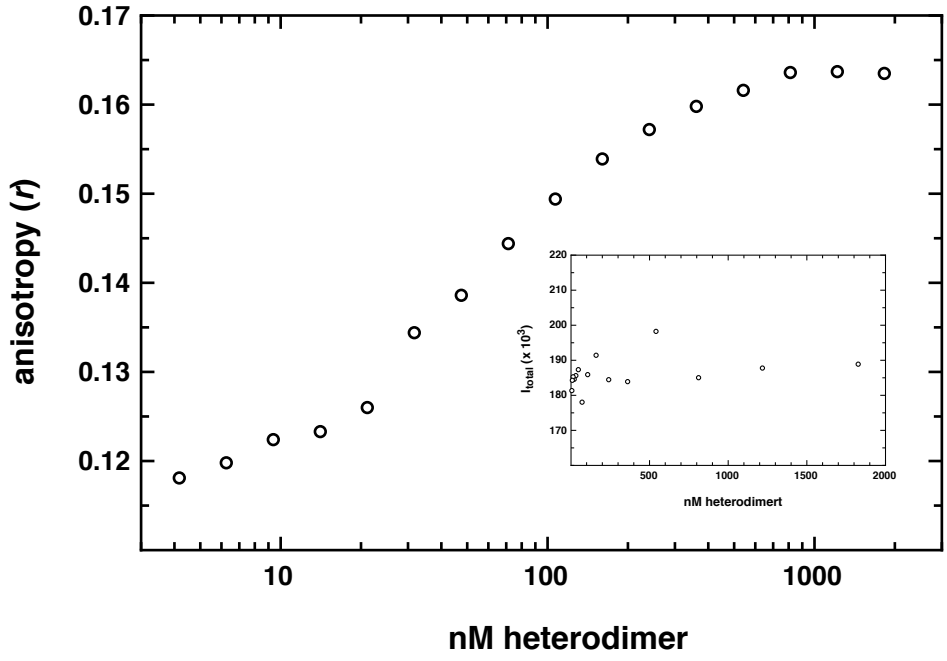

Fig 7: Binding of a 28 kDa obligate heterodimer to Alexa Fluor 488 labelled 109 amino acids long peptide. The insert shows the total intensity ($I_{\parallel} + 2I_{\perp}$) as a function of the heterodimer concentration. Excitation was at 485 nm and emission at 520 nm.

fluorescence anisotropy. Once the preliminary experiment is satisfactory it should be repeated at least three times, the average and standard deviation (not standard error) calculated, and the results plotted (Fig 9). With little imagination, this method can be easily converted to be used in different format plates (e.g., 384 wells plate) and different makes of plate readers. If necessary, it can also be adapted for use in spectrofluorometers. For different fluorophores with different excitation and emission wavelengths the rotational correlation time of the free fluorophores is calculated from their molecular weight using the Stokes-Einstein-Debye equation. Their theoretical anisotropy can then be calculated from the Perrin equation.

With practice, each experiment takes about 30 minutes to prepare and about 5 to 15 minutes to run (not including equilibration). Thus, it is possible to run a full set of experiments in less than half a day. Providing the data is of reasonable quality, the analysis does not take long and provides a satisfactory measurement of the K_d.

3. Data Analysis

In this section fluorescence anisotropy data is analysed using a one-to-one binding model and non-linear regression analysis to calculate the dissociation equilibrium constant (K_d). Other types of data analysis, such as folding/unfolding and competition assays, are dealt with elsewhere. We routinely use 'pro Fit' software (http://www.quansoft.com) for non-linear regression analysis. Other vendors of non-linear regression analysis software are available in the marketplace.

3.1 Binding of a macromolecule to a fluorescently labelled ligand – basic theory.

Consider the following simple reaction scheme where a macromolecule, say a protein, *P*, binds to a fluorescently labelled ligand, *L*, to form the complex *PL*

Eq 3.1 $$P + L \xrightarrow{k_{on}} PL$$

Where k_{on} is the forward rate constant.

The equilibrium association constant, K_a, for this reaction is described by the following expression:

Eq 3.2 $$K_a = \frac{[PL]}{[P][L]}$$

The dissociation of the complex *PL* is expressed in the following reaction scheme:

Eq 3.3 $$PL \xrightarrow{k_{off}} P + L$$

Where k_{off} is the reverse rate constant.

The equilibrium dissociation constant, K_d, for the reverse reaction is described by the following expression:

Eq 3.4 $$K_d = \frac{[P][L]}{[PL]}$$

From equations 3.2 and 3.4 it follows that K_d is the inverse of K_a.

Eq 3.5 $$K_d = \frac{1}{K_a}$$ and Eq 3.6 $$K_a = \frac{1}{K_d}$$

The fractional saturation of *L* (θ), for a one-to-one binding model can be expressed as:

Eq 3.7 $$\theta = \frac{[PL]}{[L]+[PL]}$$

Where θ varies from zero to one.

Rearranging equation 3.2 to,

Eq 3.8 $$[PL] = K_a[P][L]$$

Substituting equation 3.7 with equation 3.8 resilts in,

Eq 3.9 $$\theta = \frac{K_a[P][L]}{([L]+K_a[P][L])} = \frac{K_a[P][L]}{(1+K_a[P])[L]} = \frac{K_a[P]}{1+K_a[P]}$$

From equation 3.6 K_a is equal to $1/K_d$. Substituting this equality into equation 3.9 gives:

Eq 3.10 $$\theta = \frac{\frac{[P]}{K_d}}{1+\frac{[P]}{K_d}} = \frac{[P]}{K_d+[P]}$$

Equation 3.10 describes the Langmuir isotherm and a plot of the fractional saturation, θ, against the free (unbound) protein, gives a hyperbolic curve for a one-to-one interaction.

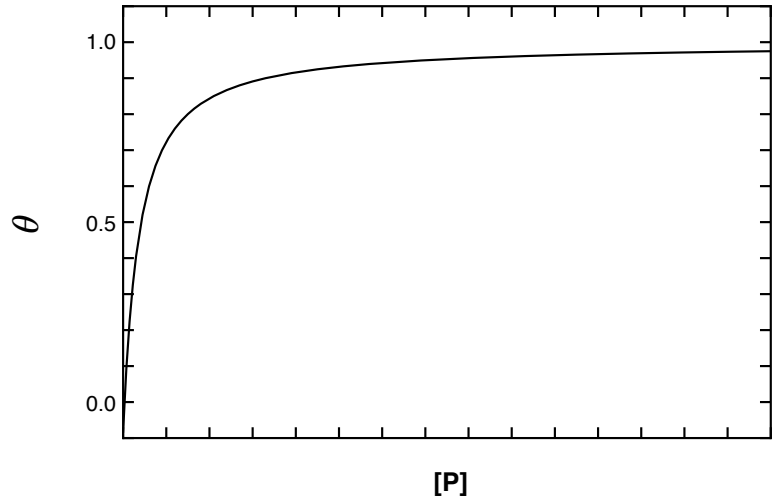

Fig 8: Langmuir isotherm where the fractional saturation is plotted against the unbound protein.

When θ equal 0.5 (half saturation), K_d is equal to $[P]_{0.5}$.

Eq 3.11 $$\theta = \frac{1}{2} = \frac{[P]_{0.5}}{K_d+[P]_{0.5}}$$

and,

$$K_d + [P]_{0.5} = 2[P]_{0.5}$$
$$\therefore K_d = [P]_{0.5}$$

3.2 Practical consideration for fluorescence anisotropy.

In fluorescence anisotropy, the anisotropy of the fluorescently labelled ligand, *L*, is denoted by r_{min}. The anisotropy of the fluorescently labelled ligand when fully saturated with the macromolecule, *P*, is denoted by r_{max}. The difference between r_{max} and r_{min} is Δr:

Eq 3.12 $$r_{max} - r_{min} = \Delta r$$ and Eq 3.13 $$\theta = \frac{(r_{obs} - r_{min})}{\Delta r}$$

Where r_{obs} is the anisotropy measured at a particular concentration of *P* and θ is the fractional saturation of *L*.

One can safely assume that r_{obs} depends linearly on the bound macromolecule *P*, and that r_{obs} is a measure of the anisotropy contribution of the unbound ligand and bound ligand *L*, such as:

Eq 3.14 $$r_{obs} = r_L[L] + r_{PL}[PL]$$

Where $[L]$ is the concentration of the unbound fluorescently labelled ligand, [PL] is the concentration of the bound fluorescently labelled ligand, and r_L and r_{PL} are constant coefficients related to the anisotropies of the unbound and bound ligand respectively.

We can adapt the Langmuir isotherm to fluorescence anisotropy experiments by substituting equation 3.13 into equation 3.10:

Eq 3.15 $$\frac{(r_{obs} - r_{min})}{\Delta r} = \frac{[P]}{K_d + [P]}$$

And rearranging equation 3.15 to:

Eq 3.16 $$r_{obs} = r_{min} + \frac{\Delta r[P]}{K_d + [P]}$$

In fluorescence anisotropy we cannot directly measure the concentration of the unbound macromolecule, $[P]$. However, in many fluorescence anisotropy experiments the amount of the bound macromolecule is a very small percentage of the total macromolecule concentration $[P]_0$, and the total macromolecule concentration can be used as an approximation of the unbound macromolecule concentration ($[P] \cong [P]_0$). Generally, if the bound macromolecule is less than 10% of the total macromolecule concentration, and the K_d is 10 folds larger than the total concentration of the fluorescently labelled ligand ($[L]_0$), the approximation holds[4]. Therefore, in order to simplify data analysis we assume that $[P] = [P]_0$ and when $[P]_0 \gg ([L]_0$, equation 3.16 becomes:

Eq 3.17 $$r_{obs} = r_{min} + \frac{\Delta r[P]_0}{K_d + [P]_0}$$

Thus, by plotting r_{obs} as a function of the total macromolecule concentration and performing a nonlinear fit to the raw data K_d can be obtained.

When the brightness of the fluorophore attached to the ligand is weak it is necessary to increase the concentration of the fluorescently labelled ligand, $[L]_0$ (section 2.1). In addition, when the K_d is close to the increased concentration of the fluorescently labelled ligand, significant amount of the macromolecule is bound ('Ligand Depletion' problem) and the unbound macromolecule concentration, $[P]$, cannot be assumed to equal the total macromolecule concentration, $[P]_0$. In such

cases, equation 3.17, which assumes that only a tiny fraction of the macromolecule is bound, cannot be used[5]. There are several methods to correct for ligand depletion. The most obvious method is to calculate the unbound macromolecule concentration, $[P]$, by subtracting the bound macromolecule, $[P]_b$ from the total macromolecule concentration, $[P]_0$. This is possible because the bound macromolecule concentration is equal to the bound fluorescently labelled ligand, $[L]_b$, which is related to the fractional saturation, θ, of L.

Eq 3.18 $$[L]_0 \times \theta = [L]_b = [P]_b$$

And

Eq 3.19 $$[P] = [P]_0 - [L]_0 \times \theta = [P]_0 - [L]_0 \frac{(r_{obs} - r_{min})}{\Delta r}$$

The calculated $[P]$ values can then be used in equation 3.16 and K_d obtained in the usual manner.

This intuitive approach is a legitimate method to obtain K_d, but there are problems associated with it. First, since the bound and free concentrations are calculated from the raw data, errors associated with measurements of the raw data are not accounted for; second, the effect of non-specific binding[3] cannot be addressed properly using this method. Kenakin[5] and Swillen[6] derived an expression that deals with 'ligand depletion' and this can be adapted to fluorescence anisotropy experiments.

The standard rate equation can be used to generate an expression that deals with 'ligand depletion'.

Eq 3.20 $$\frac{d[PL]}{dt} = k_{on}[L][P] - k_{off}[PL]$$

and Eq 3.21 $$[L] = [L]_0 - [PL]$$ Eq 3.22 $$[P] = [P]_0 - [PL]$$

Substituting equations 3.21 and 3.22 in equation 3.20 gives:

Eq 3.23 $$\frac{d[PL]}{dt} = k_{on}([L]_0 - [PL])([P]_0 - PL) - k_{off}[PL]$$

At equilibrium,

Eq 3.24 $$\frac{d[PL]}{dt} = 0$$

and,

Eq 3.25 $$k_{on}([L]_0 - [PL])([P]_0 - [PL]) = k_{off}[PL]$$

Equation 3.25 can be rearranged to give:

Eq 3.26 $$[PL]^2 - [PL]([P]_0 + [L]_0 + K_d) + [P]_0[L]_0 = 0$$

The solution to this quadratic equation is:

Eq 3.27 $$[PL] = \frac{([P]_0+[L]_0+K_d)-\sqrt{([P]_0+[L]_0+K_d)^2-4[P]_0[L]_0}}{2}$$

From equation 3.19 it can be shown that:

Eq 3.28 $$[PL] = [L]_0\theta = [L]_0\frac{r_{obs}-r_{min}}{\Delta r}$$

Substituting equation 3.28 into equation 3.27 gives the following expression which can be used to solve fluorescence anisotropy binding isotherms when 'ligand depletion' needs to be accounted for:

Eq 3.29 $$r_{obs} = r_{min} + \Delta r\frac{([P]_0+[L]_0+K_d)-\sqrt{([P]_0+[L]_0+K_d)^2-4[P]_0[L]_0}}{2[L]_0}$$

Linear regression analysis of a macromolecule binding to a 4 nM fluorescently labelled ligand (Fig 9) using equation 3.29 produced a K_d value of 32 nM, which is 8 times larger than the concentration of the labelled specie ($[L]_0$). When the difference between the K_d and the labelled specie concentration is smaller than 5 folds, equation 3.29 becomes less reliable, and it is necessary to reduce the labelled specie concentration. When the labelled specie concentration cannot be reduced due to technical difficulties, such as fluorophore brightness and detector sensitivity, it is necessary to use other methods to determine the K_d. Thus, fluorescent anisotropy is generally not a useful method for measuring sub-nanomolar K_d.

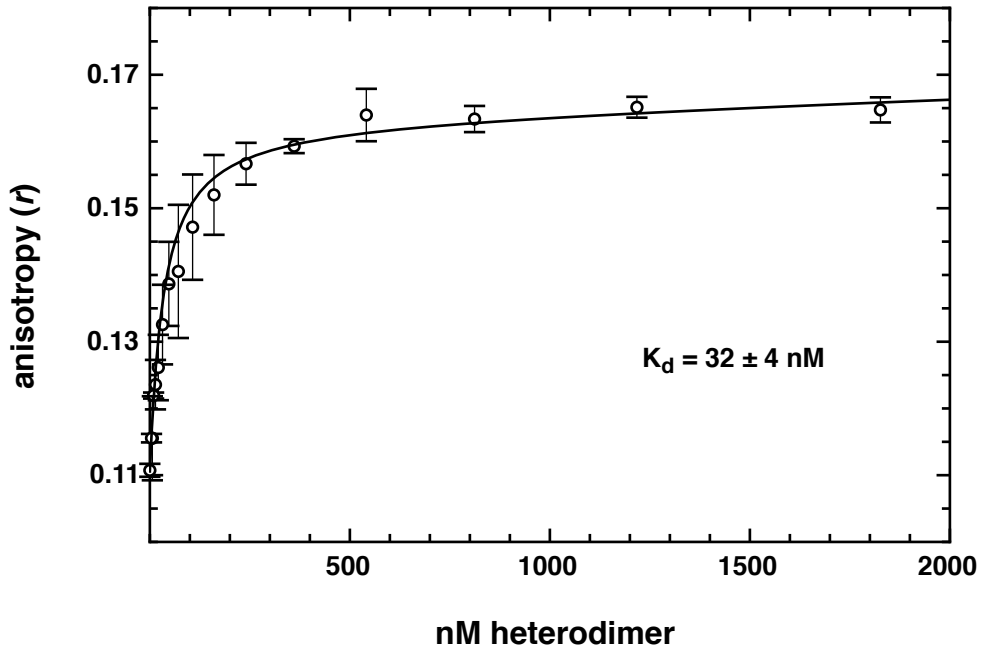

Fig 9: Binding of a 28 kDa obligate heterodimer to Alexa Fluor 488 labelled 109 amino acids long peptide. The error bars represent one standard deviation (1 SD) of 4 independent repeats. The line through the data points represents the non-linear fit. The 'goodness of fit' was 0.99, the Chi squared was 5.1367 (milli anisotropy) and the 95% confidence interval was K_d of 26 to 41 nM (17 data points; 13 degrees of freedom). Excitation was at 485 nm and emission at 520 nm.

3.3 Correcting for changes in fluorophore brightness.

If changes to the fluorophore brightness are observed when the fluorescently labelled ligand binds to the macromolecule (section 2.1), the equation for fractional saturation has to be modifies by the factor Q, which is the ratio of the observed total intensity ($I_{\parallel} + 2I_{\perp}$) and the total intensity of the free fluorescently labelled ligand.

Eq 3.30 $$Q = {I_{obs}}/{I_{free}}$$

The equation for correcting for changes in the total fluorescence intensity is[7]:

Eq 3.31 $$\theta = \frac{(r_{obs} - r_{min})}{\Delta r + (Q-1)(r_{max} - r_{obs})}$$

Where, θ, is the fractional saturation. Rearrangement of equation 3.29 to convert it to fractional saturation gives:

Eq 3.32 $$\theta = \frac{r_{obs} - r_{min}}{\Delta r} = \frac{([P]_0 + [L]_0 + K_d) - \sqrt{([P]_0 + [L]_0 + K_d)^2 - 4[P]_0[L]_0}}{2[L]_0}$$

References:

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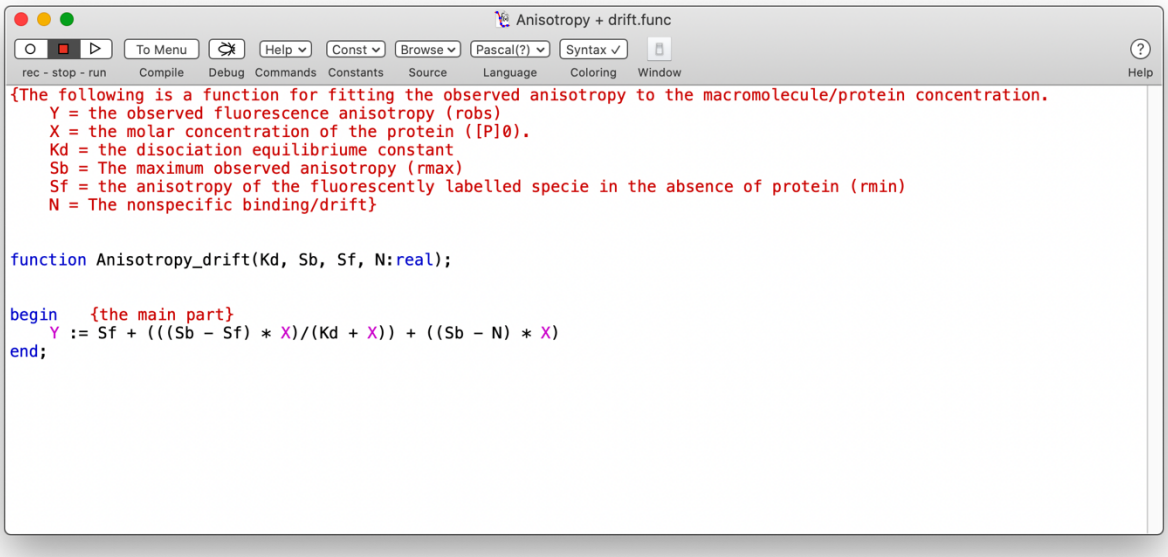
Appendix I:

This appendix contains 3 pro-Fit functions that we use for non-linear regression of fluorescence anisotropy data. These mini programs are written in Pascal + but can be adapted to the syntax any coding language. It is important to maintain the exact syntax as is shown in the figures for use in pro-Fit. Initial curve fitting should be by Monte Carlo algorithm in order to avoid the fit ending in the first Chi squared minimum. Once the best (lowest) Chi squared value is reached and is not changing, the Monte Carlo algorithm can be stopped. Use the fitted parameters from the Monte Carlo fit for the Levenberg-Marquardt algorithm where the error can be analysed. Please read the pro-Fit manual if you want to gain a better understanding of it.

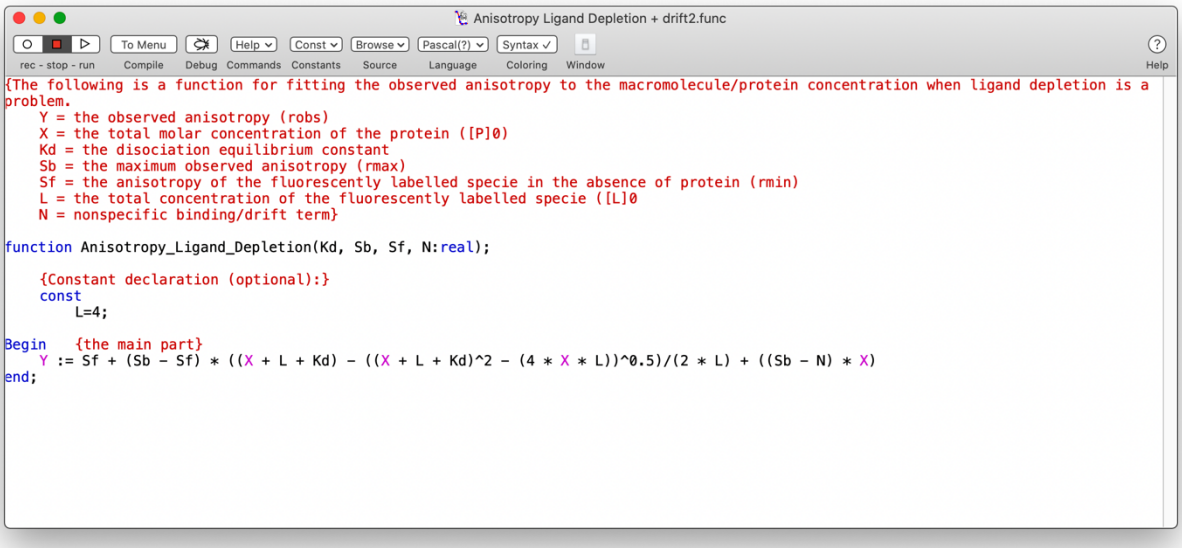
Program 1:

This program is used to fit anisotropy data using equation 3.17. The last term in the program equation, ((Sb – N) * X), is a mathematical drift term that accounts for non-specific binding and scattering. It has no physical meaning and is purely used to improve the fit.



Program 2:

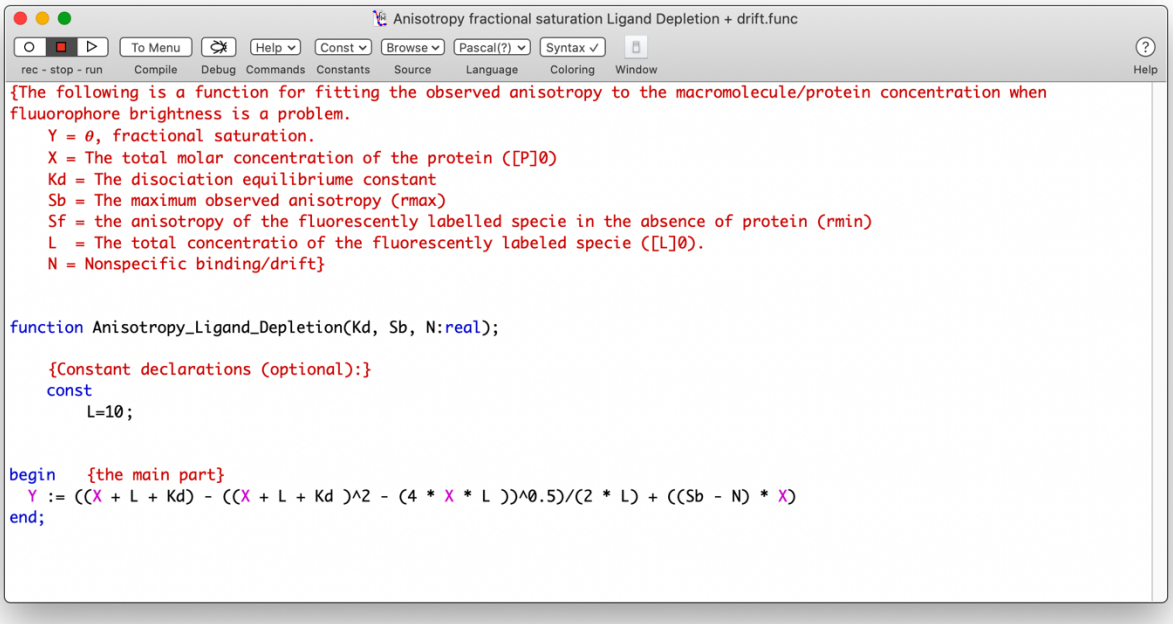
This program is used to fit anisotropy data when 'ligand depletion' is a problem, using equation 3.29. The last term in the program equation, ((Sb – N) * X), is as in program 1.




```
{The following is a function for fitting the observed anisotropy to the macromolecule/protein concentration when ligand depletion is a
problem.
    Y = the observed anisotropy (robs)
    X = the total molar concentration of the protein ([P]0)
    Kd = the disociation equilibrium constant
    Sb = the maximum observed anisotropy (rmax)
    Sf = the anisotropy of the fluorescently labelled specie in the absence of protein (rmin)
    L = the total concentration of the fluorescently labelled specie ([L]0
    N = nonspecific binding/drift term}

function Anisotropy_Ligand_Depletion(Kd, Sb, Sf, N:real);

    {Constant declaration (optional):}
    const
        L=4;

Begin    {the main part}
    Y := Sf + (Sb - Sf) * ((X + L + Kd) - ((X + L + Kd)^2 - (4 * X * L))^0.5)/(2 * L) + ((Sb - N) * X)
end;
```

Program 3:

This program is used to fit anisotropy data when fluorophore brightness is a problem, using equation 3.32. The last term in the program equation, ((Sb – N) * X), is as in program 1.




```
{The following is a function for fitting the observed anisotropy to the macromolecule/protein concentration when
fluuorophore brightness is a problem.
    Y = θ, fractional saturation.
    X = The total molar concentration of the protein ([P]0)
    Kd = The disociation equilibriume constant
    Sb = The maximum observed anisotropy (rmax)
    Sf = the anisotropy of the fluorescently labelled specie in the absence of protein (rmin)
    L  = The total concentratio of the fluorescently labeled specie ([L]0).
    N = Nonspecific binding/drift}


function Anisotropy_Ligand_Depletion(Kd, Sb, N:real);

    {Constant declarations (optional):}
    const
        L=10;


begin    {the main part}
  Y := ((X + L + Kd) - ((X + L + Kd )^2 - (4 * X * L ))^0.5)/(2 * L) + ((Sb - N) * X)
end;
```