CHAPTER 8
Analysis of FP Binding Data

Determination of Binding Constants

Definitions

"**Receptor**" and "**ligand**" have traditionally been used to describe large, membrane-bound receptors and small, soluble ligands, respectively. In fact, the terms are interchangeable when discussing any two molecules that bind to each other; the analysis of binding data does not draw a distinction between the two terms. In most traditional binding experiments, the receptor concentration is kept constant and the ligand concentration is varied. It is equally correct to keep the ligand concentration constant and vary the receptor concentration. In FP, on the other hand, it is the small, labeled ligand that is held constant at low concentrations and a much larger, unlabeled molecule is titrated against it. The experiments are done this way in order to vary the labeled ligand from the completely free state (and lowest polarization value) to the completely bound state (and highest polarization value, thus maximizing the dynamic range of the experiment. The role of the receptor and ligand in FP experiments is opposite that of traditional radioligand experiments (where [receptor] is held constant and [ligand] varies). The scientist using fluorescence polarization must, therefore, decide whether to use the classic equations as written and hope to remember that receptor really means ligand (and *vise versa*), or instead rewrite those equations for experiments using fluorescence polarization.

In past Editions of this Guide, we had chosen the former route – to the endless confusion of our readers. In this Edition, we have taken then second route, rederiving these equations specifically for FP-based applications. We still retain the intuitive phrases "ligand" to refer to the small, labeled molecule, and "receptor" for the much larger, unlabeled molecule.

Polarization vs. Anisotropy

Polarization and anisotropy are both derived from the measured vertical and horizontal intensities (see **Chapter 1**). The values are mathematically related and easily interconverted. Both values represent a weighted average of the bound vs. unbound states of the fluorescent molecule. If most of the ligand is unbound, the polarization/anisotropy value will be low. As the fraction of labeled molecules that is bound increases, the polarization/anisotropy value increases to a maximum value that corresponds to the fluorescent molecule being 100% bound.

A population of excited, identical molecules in solution all have the same polarization value. If a portion of these molecules undergo an apparent size change such that their rotational relaxation rate also changes, the observed polarization value represents an average of the component polarizations of all of the molecules. The polarization of a mixture of molecules is given by (Weber, 1952):

Equation 8.1:

$$\left(\frac{1}{P}-\frac{1}{3}\right)^{-1}=\sum_{i=1}^{n} f_i\left(\frac{1}{P_i}-\frac{1}{3}\right)^{-1}$$

where each fluorophore species has a polarization P_i and fractional fluorescence intensity f_i. On the other hand, the additivity of anisotropies is given by a simpler equation:

Equation 8.2:

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

• Weber, G. (1952) *Biochem.* **51:**145-55.

Due to mathematical simplicity, anisotropy values are preferred because it is easier to deconvolute anisotropy values into their component values than it is polarization values. It should be noted again that in the majority of applications, anisotropy does not give any additional information than polarization.

As shown in **Table 8-1**, polarization values can be manipulated as if they were anisotropy values (*i.e.*, combined by simple additivity); the resultant errors are in the range of less than 1% to 2.5% when polarization values reach 250 mP.

Table 8-1. The polarization value of a hypothetical mixture was calculated using Equation 8.1 by treating the polarization values as though they were anisotropies (simply additive).

		Fraction	Using Equation 8.3	Using Simple Additivity	Error
Example 1					
Free Ligand	50 mP	80%			
			71 mP	70 mP	< 1%
Bound Ligand	150 mP	20%			
Example 2					
Free Ligand	50 mP	60%			
			133 mP	130 mP	2.5%
Bound Ligand	250 mP	40%			

Since most instruments calculate both polarization and anisotropy, there is little reason to use polarization in your analysis. If your instrument does not report anisotropy values, the two are easily interconverted:

$$\textbf{Equation 8.3:} \qquad A = \frac{2 \times P}{3 - P}$$

We generally use the term "Fluorescence Polarization" instead of "Fluorescence Anisotropy" because FP is most often the term used to describe the entire technology. In many applications that involve a minimum of curve analysis, we still use polarization.

Relationship of Anisotropy to Bound/Free Ratio

Fluorescent molecules involved in binding events (*e.g.* ligand-receptor interactions) will exist in only one of two states: bound or free. In the general case, the bound and free states of the fluorescent ligand will each have a unique anisotropy value - high for the bound state and low for the free state (quenching caused by binding complicates the analysis). With only two species, the anisotropy additivity equation reduces to:

$$\textbf{Equation 8.4:} \qquad A = F_f A_f + F_b A_b$$

where: $F_f + F_b = 1$

A = observed anisotropy value

F_f = fraction of fluorescent ligand that is free

F_b = fraction of fluorescent ligand that is bound

A_f = anisotropy of the free fluorescent ligand

A_b = anisotropy of the bound fluorescent ligand

The top and bottom plateaus of the semi-log equilibrium binding isotherm (anisotropy vs. log total receptor concentration) define the anisotropy of the free and bound states, A_f and A_b. With the observed anisotropy, A, we can calculate the fraction of Bound and Free fluorescent ligand for a given anisotropy value. For instance, if the A_{free} = 60 mA (mA = millianisotropy units) and A_{bound} = 160 mA, at a mA = 110, half of the fluorescent ligands will be bound and half will be free. At A = 135 mA, 75% of the fluorescent ligands will be bound. This linearity of response means that anisotropy values can be equated directly to 'fraction bound,' though it may be more prudent to convert anisotropy values to bound ligand concentration. This is especially true for the proper analysis of direct equilibrium binding experiments, when the value of bound ligand is required to calculate the concentration of bound and free unlabeled receptor.

Changes in Fluorescence Intensity and Fluorescence Lifetime

The simple use of polarization or anisotropy data is predicated on the quantum yield of the fluorophore being the same in the bound (Q_b) and free (Q_f) state. Changes in quantum yield of the fluor is usually not a problem unless the fluor is directly involved in the binding event, for instance, with the binding of fluorescein by a anti-fluorescein antibody. **Chapter 3** describes the mathematical corrections necessary when quenching occurs.

Seeing a Signal

In order to see a change in polarization, the concentrations of ligand and receptor concentrations must be chosen so that at low receptor concentrations, the ligand remains unbound (low polarization value), and at high receptor concentrations, the ligand is primarily bound (high polarization value). Careful choice of the experimental concentrations will maximize the difference between the highest and lowest polarization values and therefore increase the sensitivity of the assay.

Solving the general **Equation 7.2** for B (receptor:ligand complex) in terms of R_T and L_T yielded **Equation 7.8**, presented in this chapter as **Equation 8.5**:

Equation 8.5:

$$B = \frac{L_T + K_d + R_T - \sqrt{[(L_T + K_d + R_T)^2 - 4L_TR_T]}}{2}$$

Notice that **Equation 8.5** is perfectly symmetrical with respect to R and L, as stated at the beginning of this chapter. That is, R_T and L_T are interchangeable. Experimentally, either the ligand or receptor can be held constant and the other varied.

The explicit solution of B in terms of R_T and L_T, and the obvious interexchangeability of R_T and L_T is a striking demonstration that the analysis of FP data is completely analogous to the analysis of classical radioligand binding experiments. The implications of **Equation 8.5** with regards to the percentage of ligand and receptor bound at different concentrations are shown in **Table 8-2**.

Table 8-2. Range of percentages of ligand and receptor bound under typical experimental conditions.

Ligand Concentration ([L]/K_d)	Receptor Concentration ([L]/K_d)	% Ligand Bound at Equilibrium	% Receptor Bound at Equilibrium
0.01	0.01	1	1.0
	0.1	9	0.5
	1	50	0.1
	10	91	0.09
	50	98	0.02
0.1	0.01	0.9	9.0
	0.1	8.4	8.4
	1	49	4.9
	10	91	0.9
	50	98	0.2
1	0.01	0.1	50
	0.1	5.0	49
	1	38	38
	10	90	9.0
	50	98	2.0
10	0.01	0.1	91
	0.1	0.9	90.8
	1	9	90.1
	10	73	73
	50	98	19.5

We would normally design FP binding experiments so that the labeled ligand concentration would be kept well below the K_d ([L]/K_d < 0.1). As receptor is titrated against ligand, the percentage of ligand bound varies across a wide percentage (*i.e.,* large signal dynamic range). Simultaneously, very little receptor is bound, thus avoiding a situation of "receptor depletion" (analogous to the "ligand depletion" described in **Chapter 7**).

If the FP experiment is run at a higher constant ligand concentration, as occurs in many cases, the percent of the ligand bound versus receptor concentration still covers an adequate range, but significant amounts of receptor are also bound ("receptor depletion"). In this case, the simplified binding equations, which assume only a tiny fraction of receptor is bound, cannot be used. The explicit equations must be used instead. The determination of K_d and the solution of binding equations must be dealt with mathematically as described in **Chapter 7** and later in **Chapter 8**. In the case of direct binding and competition experiments, unless the correct mathematical treatment is applied, the observed EC_{50} or IC_{50}, respectively, will be overestimations of the true K_d and K_i.

It is fortuitous that in FP competition experiments, since the receptor concentrations must be necessarily high to bind significant fluorescent ligand (the maximizing the polarization value), the "receptor depletion" effect is reduced (*e.g.* [ligand] = 1 × K_d and [receptor] = 10 × K_d, so that 90% of the ligand is bound, but only 9% of the receptor; **Table 8–2**).

Equilibrium Binding - Experimental Design

Incubation Time to Reach Equilibrium

Before a classic receptor/ligand equilibrium binding experiment can be completed, the incubation period required for equilibrium to be achieved must be empirically determined. Time to reach equilibrium is dependent on the concentrations of the ligand and receptor, so the worst case concentrations should be used, namely, the chosen constant labeled ligand concentration and the lowest receptor concentration envisioned.

Step 1. Determination of Equilibrium Time using the Beacon® 2000 System

1. Background blank the buffer.
2. Add the ligand to the tube and take several readings to determine the polarization of free ligand.
3. To the same tube, add an amount of receptor approximately equal to 1/20 of the expected K_d. Because the rate of association is dependent on the receptor concentration, this combination of low ligand and receptor concentrations represents the longest time necessary to reach equilibrium.
4. Follow the increase in polarization over time (begin with 10 minute intervals). Determine the time at which the polarization values plateau, representing the approach to equilibrium. Use this time for the incubation period of the binding experiments outlined later.

Constructing a Binding Isotherm

As described earlier, the format of the typical equilibrium FP binding experiment involves incubating a sub-K_d concentration of labeled ligand with a wide range of receptor concentrations from below to above the anticipated K_d. In those cases when there is no prior information about the K_d, it is not disastrous to incorrectly choose these concentrations; it will become apparent when the data are inspected if the experiment should be repeated with a different range of receptor concentrations.

Step 2. Construction of a Binding Isotherm Using the Beacon® 2000 Analyzer

1. Serially dilute your unlabeled receptor into approximately 20 test tubes, covering the range of concentrations from 10- to 20-fold below the K_d to approximately 50- to 100-fold above the K_d. The final volumes should be at least 0.1 mL.
2. If the background fluorescence of the tube with the highest concentration of receptor is very low, there is no need to account for the background fluorescence. The background contributed by some samples (*e.g.*, from impure protein or antibody preparations) can be high and must be taken into account. There are two suggested correction methods:
    a. If polarization values will be determined only once, a single set of tubes is sufficient (as described above). Measure and record the background intensity of each tube with the Beacon® 2000 Fluorescence Polarization System in its batch mode and then continue on to **Step 3**.
    b. In some experiments, it may be useful to determine polarization values several times, for instance, after incubations at different temperatures. Background fluorescence values need to be determined each time the samples are reread. If the labeled ligand has already been added to the tubes, measurement of background fluorescence is impossible. In these cases, prepare a duplicate set of tubes that will not receive the fluorescent ligand.
3. Add identical aliquots of the ligand to each tube and mix.
4. Incubate the tubes for the time determined in **Step 1**.
5. Measure the polarization value of each tube. Because the Beacon® 2000 Fluorescence Polarization System is being used in the batch mode, it is important that determinations are made in the same order as blank readings were taken.

Analysis of Binding Constants

Upon completion of the binding experiment, the measured data will be millipolarization (or millianisotropy) units vs. total receptor concentration. Much was said in the previous chapter about analysis of binding curves. In this Section, we will limit our discussion to only the practical considerations of analysis of these data.

Step 3. Convert to Anisotropy

If you didn't already record your data as anisotropy, now is the time to convert the polarization values. Either acquire the anisotropy data from your downloaded Beacon® 2000 Fluorescence Polarization System spreadsheet or convert your polarization values to anisotropy.

Step 4. Receptor Depletion?

Before choosing the equations to use for analysis, it is important to know whether receptor depletion is a problem. That is, is a significant percentage of the receptor is bound such that the total receptor concentration cannot be used as a good approximation of the free receptor concentration. In determining if there is a problem, the mA values must first be converted into bound ligand concentrations using the following equation:

$$\textbf{Equation 8.6:} \qquad [\text{Ligand Bound}] = L_T \times \frac{A - A_f}{A_b - A_f} = B$$

where:

L_T = the total added concentration of ligand
A = the experimental anisotropy
A_f = the anisotropy for the free ligand
A_b = the anisotropy for the fully bound ligand

The quotient is termed F_b and is equal to the fraction of ligand bound. For this application, the values of A_b and A_f can be visually estimated or determined by a curve-fitting program. Later we will want precise values for these parameters.

For each receptor concentration we now have a corresponding concentration of bound ligand (really the receptor:ligand complex), and it is easy to compare these values to determine what percentage of receptor is bound in the complex. If the bound receptor:ligand complex concentration is greater than 10% of total receptor concentration, we should not substitute the total receptor concentration for the free receptor concentration.

Step 5. Analysis of Binding Isotherm with Receptor Depletion

As stated in the previous chapter, there are several methods to correct for receptor depletion. Probably the most obvious method is to calculate the free receptor concentration by subtracting the bound receptor from total receptor since you know both of these values. Most people do this, and it is a legitimate method, but as discussed in **Chapter 7** there are problems with this approach. First, bound and free concentrations, and their errors, are related, and second, the effect of non-specific binding cannot be addressed properly (luckily, NSB is almost never present in any measurable degree in FP-based experiments, because the technique is solution-based). The intuitive method described above will work, but we encourage all readers to seek out the references mentioned in **Chapter 7** for a more complete explanation of the binding analysis in the presence of receptor depletion. The decision to calculate free receptor concentrations by subtraction or to use the explicit equations below is best left to the researcher and their unique situation.

Following the logic in **Chapter 7**, we derived the equation for the binding isotherm which can be rearranged to:

$$\textbf{Equation 8.7:} \qquad B = \frac{R_F \times L_T}{K_d + R_F}$$

If we choose not to estimate R_F, the free receptor concentration, with R_T, the total receptor concentration, we must substitute the term $(R_T - B)$ for R_F.

$$\textbf{Equation 8.8:} \qquad B = \frac{L_T(R_T - B)}{K_d + (R_T - B)}$$

One solution for B in **Equation 8.8** is:

Equation 8.9:

$$B = \frac{L_T + K_d + R_T}{2} - \frac{\sqrt{(-L_T - K_d - R_T)^2 - 4L_TR_T}}{2}$$

This is the equation Kenakin (1993) essentially gives as his Eq. 12.14, and Swillens (1995) as his Eq. 3 (with a non-specific binding component), and Lundblad *et al.* (1996) as their Eq. 8. This equation can be solved by a non-linear regression program yielding estimates for K_d and L_T. If you would rather use the anisotropy data directly without conversion to bound receptor/ligand values, substitute **Equation 8.9** for B in **Equation 8.6** and solve for A, the measured anisotropy.

Equation 8.10:

$$A = A_f + (A_b - A_f) \times \frac{(L_T + K_d + R_T) - \sqrt{(L_T - K_d - R_T)^2 - 4L_TR_T]}}{2L_T}$$

L_T is known and A is measured for each R_T. The equation can now be solved for K_d, A_b, and A_f.

Step 6. Analysis of Binding Isotherm without Receptor Depletion

If receptor depletion is not a problem, these equations can be simplified because R_T is a good approximation of R_F.

Equation 8.11:

$$B = \frac{R_T \times L_T}{K_d + R_T}$$

Again, this equation can be solved by computer assisted non-linear regression if the anisotropy data are converted to bound receptor:ligand complex, B, or the anisotropy data can be fitted directly. In a manner analogous to the derivation of **Equation 8.10**, we get:

Equation 8.12:

$$A = A_f + \left[(A_b - A_f) \times \frac{R_T}{K_d + R_T}\right]$$

which will yield values for K_d, A_f, and A_b.

- Kenakin, T.P. (1993) Pharmacologic analysis of drug-receptor interaction. New York:Raven. 483 p.
- Lundblad, J.R. *et al.* (1996) *Mol. Endo.* **10:**607-12.
- Swillens, S. (1995) *Mol. Pharm.* **47:**1197-1203.

Competition Experiments

General Considerations

Fluorescence Polarization competition experiments necessitate high receptor concentrations in order to bind significant amounts of the fluoresceinated tracer and therefore cause a shift in the polarization value. If the [receptor]/K_d ratio is ≤ 0.1, according to **Table 8-2** no more than 10% of the ligand will be bound, and the starting polarization value will still be only 10% of the maximum value. Any drop in polarization caused by the addition of an unlabeled competitor will be limited to only 10% of the possible dynamic range.

Instead, FP competition experiments should be designed such that the [receptor]/K_d ratio is about 1, so that the starting polarization value will represent 50% of the maximal shift (see **Table 8-2**). Indeed, we recommend choosing conditions such that the shift is approximately 80%. Under these conditions, though, more inhibitor is required to see a 50% drop in the amount of bound ligand compared to when the [receptor]/K_d = 0.1. The result is that the observed IC_{50} will be an overestimation of the true K_i.

Receptor-Ligand Competition Experiments

Step 1. Determine the Minimum Incubation Time Necessary to Reach Equilibrium.

Presumably, this information was determined previously for the direct binding experiment. If not, some estimate of the time needed to reach equilibrium is required. Ideally, a competition experiment using a low concentration of competitor can be followed over time (reading the set of tubes at several different times until polarization values plateau).

Step 2. Constructing a Competition Isotherm

1. Serially dilute the unlabeled competitors over a range of concentrations, with a total volume for each tube of at least 0.1 mL.
2. Measure the fluorescence background of each tube in the Beacon® 2000 Fluorescence Polarization System.
3. Add identical aliquots of the receptor:labeled ligand mixture to each tube, mix, and allow to incubate until equilibrium is established. Alternatively, the receptor and labeled ligand can be added separately if preincubation of these two components is undesirable.
4. Measure the anisotropy value of each tube.

The competition curve data, mA vs. unlabeled competitor concentration, can be analyzed by computer assisted non-linear regression yielding IC_{50} values for each of the competitors.

Kinetic Experiments

Determination of the Dissociation Rate Constant, k_{-1}

The calculation of the dissociation rate constant (k_{-1}) is commonly accomplished by binding a ligand to a receptor and measuring the rate of dissociation of receptor from the ligand. In solid-phase binding experiments, ligand is allowed to bind to immobilized receptor and then the incubation medium is quickly removed from the dish or filter and replaced with buffer. This step immediately removes any free and unbound ligand from the system (hence the term "instantaneous dilution"). As the bound ligand reaches a new equilibrium with the ligand in the buffer (zero, at time = 0), the amount of ligand bound to receptor will decrease over time.

One of the primary advantages of fluorescence polarization experiments is that bound and free molecular species do not need to be separated. In dissociation experiments, however, one must find a way to disrupt the binding equilibrium and encourage dissociation of the ligand from the receptor. One method is to add a large excess of an **unlabeled** ligand to the system so that when a labeled ligand dissociates from its partner it is unlikely that labeled ligand will rebind in the face of an overwhelming concentration of unlabeled ligand. This result effectively produces a situation of infinite dilution in relation to the labeled ligand.

1. Select labeled ligand and unlabeled receptor concentrations that will result in 50% bound ligand (50% of maximal mA value). Choose the lowest ligand concentration possible so that you can still see a signal (refer to **Table 8-2**).
2. Determine the mA_{max} of this system by allowing the mixture to reach equilibrium.
3. After the mixture has reached equilibrium, add 100X the K_d of unlabeled ligand to the tube in a small volume and follow anisotropy values over time.
4. From the mA vs. time curve, estimate the mA_{min} value by curve-fitting analysis or visual inspection.
5. Plot the $\ln[(mA_{obs} - mA_{min})/(mA_{max} - mA_{min})]$ vs. time for each time point. This will yield a straight line or curve, with terminal slope $-k_{-1}$. When $mA_{obs} = 0.5\ mA_{max}$, then $k_{-1} = 0.693/T_{1/2}$, where $T_{1/2}$ is the half-time.

This method for the determination of the dissociation constant will yield reliable results unless the binding exhibits positive or negative cooperativity. In the case of positive cooperativity, the addition of unlabeled ligand will decelerate the dissociation of the labeled ligand, resulting in an underestimation of the dissociation rate. In the case of negative cooperativity, the addition of unlabeled ligand will result in an increased dissociation rate (*e.g.*, with the insulin receptor).

Determination of the Association Rate Constant, k_1

The approximate determination of the association rate constant is dependent on $B << R_T$. In this case, our rate equations simplify to **Equation 8.13**:

Equation 8.13: $$B_t = B_e \times \{1 - e^{[-(k_1 R_T + k_{-1})t]}\}$$

where B_t is bound at time t, B_e is bound at equilibrium, R_T = [total unlabeled receptor], and t = time. When $B_t = 0.5B_e$, **Equation 8.13** simplifies to **Equation 8.14**:

Equation 8.14: $$\frac{\ln(2)}{T_{1/2}} = k_1 R_T + k_{-1}$$

where $T_{1/2}$ is the half-time of association.

The rate constants are therefore calculated by determining the $T_{1/2}$ for each curve and plotting $\ln(2)/T_{1/2}$ vs. R_T. The $T_{1/2}$ can be estimated in two ways. First, the curvilinear association curve can be analyzed by computer-assisted non-linear regression and $T_{1/2}$ calculated from the fitted curve. Alternatively, the association curve can be linearly transformed and the value of $T_{1/2}$ can be estimated graphically. This second method is now outlined:

1. Empirically select concentrations of ligand and tracer that will generate a binding association curve that can be easily resolved (*e.g.*, binding that does not reach equilibrium in less than 2 minutes). The Beacon® 2000 Analyzer is used in its kinetic mode and data points are taken every 13 seconds.
2. Repeat the association experiment at a fixed ligand concentration and 5-10 different receptor concentrations.
3. Collect data on anisotropy vs. time, for each experiment.
4. Transform the anisotropy data for each curve by subtracting the zero time anisotropy value from each subsequent value.
5. For each curve, estimate the equilibrium plateau in mA units (mA_{eq}).
6. For each curve, plot $(mA_{eq} - mA_t)/mA_{eq}$ vs. time.
7. Determine the $T_{1/2}$ for each curve as the point at which log $(mA_{eq} - mA_t)/mA_{eq} = 0.5$.
8. Plot $\ln(2)/T_{1/2}$ vs. total receptor concentration. According to **Equation 7.19** (see **Chapter 7**), this plot will yield a straight line with a slope = k_1 and the y-intercept = k_{-1}.