A General Framework for Development and Data Analysis of Competitive High-Throughput Screens for Small-Molecule Inhibitors of Protein–Protein Interactions by Fluorescence Polarization

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ABSTRACT: Equilibrium binding experiments are widely used for the accurate characterization of binding and competitive binding behavior in biological systems. Modern high-throughput discovery efforts in chemical biology rely heavily upon this principle. Here, we derive exact analytical expressions for general competitive binding models which can also explain a commonly encountered phenomenon in these types of experiments, anticooperative incomplete displacement. We explore the effects of nonspecific binding behavior and parameter misestimation. All expressions are derived in terms of total concentrations determined a priori. We discuss a general framework for high-throughput screening assays based on fluorescence polarization and strategies for assay development, sensitivity regimes, data quality control, analysis, and ranking. Theoretical findings are visualized by simulations using realistic parameter sets. Our results are the basis for the discovery of small-molecule inhibitors of the protein–protein interaction between human calcineurin and NFAT transcription factors, as discussed in the subsequent paper (31).

Large complex networks of interactions between biomacromolecules are at the very center of biological and biochemical processes in living organisms (1–5). In particular, protein–protein and protein–ligand interactions, spanning a wide range of affinities, are a focus of great interest for fundamental biological (6–8) and biophysical (9, 10) investigation and as targets for drug development and pharmacological intervention (11, 12). Recent advances in chemical biology and high-throughput (HTP) screening (13–15) have introduced the use of structurally diverse small organic molecules for the targeted manipulation of protein interactions (16, 17). For one case of enzyme–substrate interactions, we have previously defined this concept as substrate-selective enzyme inhibition (SSEI) (18), which entails the inhibition of a specific enzyme–substrate docking site with the preservation of catalytic activity toward other substrates.

Fluorescence polarization (FP) is one of the most sensitive, robust, and widely used HTP methods for the study of protein interactions and drug discovery (19–22). When a fluorophore is excited with polarized light, the emitted light is also polarized. The degree of polarization is a function of molecular properties, most specifically, Brownian molecular rotation, and, hence, can serve as a sensitive molecular sensor. HTP FP assays usually detect changes in polarization caused by changes in the molecular mass of the labeled species. Such examples include enzymatic release of the free fluorophore (23) or displacement of molecular complexes (24).

FP assays are typically designed as competitive equilibrium binding assays. For algebraic simplicity, equilibrium binding is described in most textbooks in terms of effective concentrations of the various molecular species involved (25–28), but not in terms of a priori known absolute input concentrations. This makes the direct use of the equations impractical for data fitting and parameter estimation. An exact analytical treatment of competitive binding has, to our knowledge, not previously been applied to FP experiments. Instead, numerical approximations are more commonly used (29). Only rarely has an analytical approach been attempted, and then limited, however, to detection methods other than FP (30). Furthermore, incomplete competitive displacement, as described by a four-state model, has, to the best of our knowledge, not been analyzed in this context before. Moreover, there are numerous examples in the literature in which FP data have been fitted to incorrect relationships, wrongly assuming a linear superposition principle for polarization. Anisotropy should have been used instead (vide infra), or the correct nonlinear superposition principle should have been applied.

1 Abbreviations: FP, fluorescence polarization; HTP, high-throughput; NFAT, nuclear factor of activated T cells.

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We feel that a comprehensive investigation of binding scenarios and a road map for the successful design of HTP FP assays are timely and useful. In this and a subsequent paper (31), we provide further details on our work on the selective disruption of the NFAT–calcineurin complex (31). In the paper presented here, we discuss the theoretical framework of screening assays based on FP and strategies for HTP assay development, sensitivity regimes, data quality control, analysis, and ranking. The results are general and widely applicable to equilibrium competition studies. We also provide numerical simulations for visually exploring the theoretical results and a procedural flowchart for the practical realization of HTP FP assay projects. Our results are the basis for the discovery of novel NFAT-directed small-molecule inhibitors of human calcineurin (18, 31).

1. THEORY

The following presentation focuses on direct binding of one ligand or competitive binding of two ligands to a binding partner under equilibrium conditions. We will study both complete and incomplete competitive binding and extend our models to account for nonspecific binding effects.

1.1. Direct Binding. In FP assays, a fluorescently labeled ligand, Ls, binds to an unlabeled receptor, R. If one does not account for potential nonspecific binding events, this simple interaction is described by eqs 1–4 and is illustrated in Figure 1A.

\[
K_{D1} = \frac{R L_s}{RL_s} \tag{1}
\]

\[
R_T = R + RL_s \tag{2}
\]

\[
L_{ST} = L_s + RL_s \tag{3}
\]

\[
F_{SB} = 1 - \frac{L_s}{L_{ST}} \tag{4}
\]

\[
R, L_s, \text{ and } RL_s \text{ denote free (unbound) concentrations of the receptor, labeled ligand, and labeled–receptor complex, respectively. } K_{D1} \text{ is the dissociation constant of the interaction. } R_T \text{ and } L_{ST} \text{ are total input concentrations of the species.}
\]

2 Definitions of repeatedly used variables (Note that in this paper complexes are printed in regular font, not italics): \( R \), total receptor concentration; \( R_T \), concentration of the free receptor; \( L_{ST} \), total concentration of the labeled ligand (probe); \( L_s \), concentration of the free labeled ligand (probe); \( L_T \), total concentration of the unlabeled ligand (competitor); \( L \), concentration of the free unlabeled ligand (competitor); \( RL_s \), concentration of the binary complex of the receptor and labeled ligand (probe); \( RL_T \), concentration of the ternary complex of the receptor, unlabeled ligand (competitor), and labeled ligand (probe); \( K_{D1} \), dissociation constant of the interaction between \( R \) and \( L_s \) (probe); \( K_{D2} \), dissociation constant of the interaction between \( R \) and \( L_T \) (competitor); \( K_{D3} \), dissociation constant of the interaction between the RL complex and \( L_s \); \( F_{SB} \), fraction of bound labeled ligand (probe); \( N_s \), parameter for nonspecific binding of the labeled ligand (probe); \( N \), parameter for nonspecific binding of the unlabeled ligand (competitor); \( F^* \), fluorescence intensity parallel to the plane of exciting light; \( F^\perp \), fluorescence intensity perpendicular to the plane of exciting light; \( A \), anisotropy; \( \kappa \), angle between absorption and emission dipoles; \( \lambda \), angle associated with local motion of the fluorescent moiety; \( \tau_c \), fluorescence lifetime of the fluorophore; \( \tau_i \), rotational correlation time of the observed species; \( Q \), ratio of fluorescence intensities of bound and free species.

\[
A = \frac{RL_s}{RL_T} \tag{5}
\]

\[
B = \frac{RL_s}{RL_T} \tag{6}
\]

\[
C = \frac{RL_s}{RL_T} \tag{7}
\]

\[
D = \frac{RL_s}{RL_T} \tag{8}
\]

The dependence of \( F_{SB} \) on \( R_T \) and \( K_{D1} \) is simulated and discussed in section 2.1 of this paper (Figure 2A, vide infra).

We can model nonspecific immobilization of the labeled ligand by introducing a dimensionless, non-negative parameter, \( N_s \), and modifying eq 3 to give eq 7.

\[
L_{ST} = L_s + RL_s + N_s L_s \tag{7}
\]

Here, it is assumed that, for typically available ligand concentrations, nonspecific binding is nonsaturable and linearly proportional to the free labeled ligand concentration (30).

In experiments with fluorescently labeled ligands, the total concentration of labeled ligand, \( L_{ST} \), is typically kept constant and the relationship between \( R_T \) (independent variable) and \( F_{SB} \) (dependent variable) is measured (vide infra) to extract an estimate of \( K_{D1} \). Solving eqs 1–4 for \( R_T \) and eliminating \( R, L_s, \) and \( RL_s \), we obtain eq 5.

\[
R_T = \frac{-L_{ST} F_{SB}^2 + (K_{D1} + L_{ST}) F_{SB}}{1 - F_{SB}} \tag{5}
\]

We note that eq 5 is quadratic in \( F_{SB} \). Solving eqs 1–4 for \( F_{SB} \) analogously, we find the well-known eq 6. Note that only the given root represents a physical solution.

\[
F_{SB} = \frac{K_{D1} + L_{ST} + R_T - \sqrt{(K_{D1} + L_{ST} + R_T)^2 - 4L_{ST} R_T}}{2L_{ST}} \tag{6}
\]
and

\[ b_1 = (1 + N_S)K_{D1} + L_{ST} + 2N_SL_{ST} \]

\[ F_{SB} = \frac{K_{D1} + L_{ST} + R_T + (K_{D1} + 2L_{ST})N_S - \sqrt{c}}{2(1 + N_S)L_{ST}} \]  (9)

with

\[ c = (1 + N_S)^2K_{D1}^2 + 2(1 + N_S)(L_{ST} + R_T)K_{D1} + (L_{ST} - R_T)^2 \]

In analogy to eq 5, eq 8 is quadratic in \( F_{SB} \). When \( N_S = 0 \), eqs 8 and 9 become equivalent to eqs 5 and 6, respectively, and we recover the purely specific binding case. Since \( F_{SB} \) is closely related to the spectroscopic readout of anisotropy in FP as a function of \( R_T \) (see section 1.4), data can be directly fitted using eq 6 or 9 (i.e., the measured dependent variable is expressed as a function of the independent input variable).

To perform accurate competition experiments, we will in general only pursue labeled ligands that do not display significant nonspecific binding behavior, i.e., for which we find \( N_S = 0 \). An estimate of the contribution of \( N_S \) originating from nonspecific binding to the reaction compartment or its components other than the receptor may be obtained by measuring the anisotropy of the labeled ligand in the absence of receptor. If this value is significantly greater than that and is consistent with the type of fluorophore used and the size of the labeled ligand (see section 1.4 and ref 31), one can conclude that \( N_S > 0 \). In fact, when \( R_T = 0 \) we find eq 10, from which \( N_S \) can be directly determined.

\[ F_{SB}^{(R_T=0)} = \frac{N_S}{1 + N_S} \]  (10)

Let us finally consider the effect on the estimate of \( K_{D1} \), if it were falsely assumed that \( N_S = 0 \), even though \( N_S \) is in reality greater than zero. One would then (incorrectly) conclude that \( F_{SB} = 0 \) when \( R_T = 0 \). Using eq 10, the assumed fraction of the bound labeled ligand, \( F_{SBA} \), and the true fraction, \( F_{SB} \), are then related by eq 11.

\[ F_{SBA} = (1 + N_S)F_{SB} - N_S \]  (11)

Solving for \( R_T \) in analogy to eq 8, we obtain eq 12.

\[ R_T = \frac{-aF_{SB}^2 + b_2F_{SB} - \left( \frac{1}{1 + N_S}K_{DIA} + L_{ST} \right)N_S}{1 - F_{SB}} \]  (12)

with

\[ a = L_{ST} + N_SL_{ST} \]

and

\[ b_2 = K_{D1A} + L_{ST} + 2N_SL_{ST} \]

where \( K_{DIA} \) denotes the dissociation constant that would be obtained under the assumption \( N_S = 0 \). By direct comparison of eqs 8 and 12, we find that \( K_{DIA} \) is greater than the true value, \( K_{D1} \), by a multiplicative factor of \( 1 + N_S \). Hence, \( K_{DIA} \)

is always a robust upper bound estimate of the true dissociation constant; i.e., neglecting nonspecific effects will, at worst, introduce a conservative error and lead to over-estimation of the dissociation constant. In section 2.1, numerical simulations illustrate these effects (Figure 2B, *vide infra*).

**1.2. Complete Competitive Binding.** In competitive FP assays, an unlabeled ligand, \( L \), and a fluorescently labeled ligand, \( L_S \), compete for binding to an unlabeled receptor, \( R \). Complete competitive binding means here that binding of labeled and unlabeled ligands is mutually exclusive. Not accounting for potential nonspecific binding events, this interaction scheme is described by eqs 1, 3, 4, and 13–15 and is illustrated in Figure 1B.

\[ K_{D2} = \frac{RL}{RL} \]  (13)

\[ R_T = R + RL_S + RL \]  (14)

\[ L_T = L + RL \]  (15)

\( L \), \( RL \), and \( L_T \) denote free unlabeled ligand, unlabeled ligand–receptor complex, and total unlabeled ligand input concentrations, respectively. \( K_{D2} \) is the dissociation constant of the interaction between \( L \) and \( R \). In all competitive types of experiments, complete or incomplete (*vide infra*), we further assume \( R_T > L_{ST} \). Otherwise, considerable amounts of free labeled ligand would always be present and interfere with measurements.
We note that eq 16 is cubic in \(RL\), we obtain eq 16.

L being constant, and the relationship between \(F\) (the ultimate experimental goal of competitive binding assays) an estimate of \(K\) typically done when a binding assay is developed. Solving from a direct binding experiment (see section 1.1), as is expressed by eq 17 (analogously yields a unique physically meaningful root.

FIGURE 3: (A) Complete competitive binding model without nonspecific effects (eq 17). (B) Incomplete competitive binding model without nonspecific effects (eq 27).

Ways and means to the precise determination of \(K_{D1}\) are the ultimate experimental goal of competitive binding assays (18, 31). Experimentally, \(R_T\) and \(L_{ST}\) are typically kept constant, and the relationship between \(LT\) (independent variable) and \(F_{SB}\) (dependent variable) is analyzed to extract an estimate of \(K_{D2}\). \(K_{D1}\) must have been previously obtained from a direct binding experiment (see section 1.1), as is typically done when a binding assay is developed. Solving the six equations for \(LT\) and eliminating \(R\), \(L_S\), \(RL_S\), \(L\), and \(RL\), we obtain eq 16.

\[
L_T = \frac{([K_{D1} - K_{D2}]F_{SB} + K_{D2})L_{ST}F_{SB}^2 - (K_{D1} + L_{ST} + R_T)F_{SB} + R_T}{(1 - F_{SB})F_{SB}K_{D1}}
\]  

(16)

We note that eq 16 is cubic in \(F_{SB}\). Solving for \(F_{SB}\) analogously yields a unique physically meaningful root expressed by eq 17 (30).

\[
F_{SB} = \frac{2\sqrt{(d^2 - 3e)}\cos(\theta/3) - d}{3K_{D1} + 2\sqrt{(d^2 - 3e)}\cos(\theta/3) - d}
\]  

(17)

with

\[
d = K_{D1} + K_{D2} + L_{ST} + L_T - R_T
\]

\[
e = (L_T - R_T)K_{D1} + (L_{ST} - R_T)K_{D2} + K_{D1}K_{D2}
\]

\[
f = -K_{D1}K_{D2}R_T
\]

and

\[
\theta = \arccos \left[ \frac{-2d^3 + 9de - 27f}{2\sqrt{d^2 - 3e}^3} \right]
\]

The dependence of \(F_{SB}\) on \(L_T\) and \(K_{D2}\) is numerically simulated and discussed in section 2.2 (Figure 3A, vide infra).

In analogy to eq 7, we can model nonspecific immobilization of the unlabeled ligand by introducing a dimensionless, non-negative parameter, \(N\), and modifying eq 15 to give eq 18.

\[
L_T = L + RL + NL
\]  

(18)

However, as laid out in section 1.1, we will not accommodate here nonspecific binding of the labeled ligand, since such molecules should be avoided in competitive experiments. Solving eqs 1, 3, 4, 13, 14, and 18 for \(L_T\), we obtain eq 19.

\[
L_T = \{([K_{D1} - (1 + N)K_{D2}]F_{SB} + (1 + N)K_{D2})
\]

\[(L_{ST}F_{SB}^2 - (K_{D1} + L_{ST} + R_T)F_{SB} + R_T)/(1 - F_{SB})F_{SB}K_{D1}\}
\]  

(19)

We note that eq 19 is cubic in \(F_{SB}\). Alternatively, we can solve eqs 1, 3, 13, 14, and 18 to obtain eq 20, a cubic polynomial in \(R\).

\[
R^3 + gR^2 + hR + i = 0
\]  

(20)

with

\[
g = K_{D1} + (1 + N)K_{D2} + L_{ST} + L_T - R_T
\]

\[
h = (L_T - R_T)K_{D1} + (1 + N)(L_{ST} - R_T)K_{D2} + (1 + N)K_{D1}K_{D2}
\]

\[
i = -(1 + N)K_{D1}K_{D2}R_T
\]

In analogy to eq 17 and also using eq 4, we can solve for \(F_{SB}\) and obtain eq 21.

\[
F_{SB} = \frac{2\sqrt{(g^2 - 3h)\cos(\xi/3) - g}}{3K_{D1} + 2\sqrt{(g^2 - 3h)\cos(\xi/3) - g}}
\]  

(21)

with

\[
\xi = \arccos \left[ \frac{-2g^3 + 9gh - 27f}{2\sqrt{(g^2 - 3h)^3}} \right]
\]

By a comparison of eq 16 with eq 19 and eq 17 with eq 21, it can be seen that nonspecific binding of unlabeled ligand is formally equivalent to a multiplicative increase in the specific dissociation constant \(K_{D2}\) by a factor of \(1 + N\). The notable consequence is that competitive binding experiments per se cannot directly separate the two effects. In the absence of an independent estimate of \(N\), \(K_{D2}\) fitted according to eq 16 or 17 provides, however, a reliable upper limit for the specific dissociation constant.

1.3. Incomplete Competitive Binding. We find from simulations (see section 2) that the complete competitive
binding model derived above predicts invariably $F_{SB} \to 0$ in the limit of increasingly large $L_T$; i.e., labeled ligand can always be completely displaced from the receptor by an excess of the unlabeled ligand. We have found experimentally, however, that a significant number of small-molecule ligands identified in HTS screens are not able to displace the labeled ligand entirely and are characterized by an asymptotic limit of $F_{SB}$ that is greater than zero (31, 32). We introduce here an incomplete competitive binding model (four-state model) that can account for this phenomenon. Not considering potential nonspecific binding events, this interaction scheme is described by eqs 1, 4, 13, and 22 considering potential nonspecific binding events, this interaction scheme is described by eqs 1, 4, 13, and 22.

$$K_{D3} = \frac{RLL_S}{RLL_S}$$

(22)

$$K_{D4} = \frac{K_{D2}K_{D3}}{K_{D1}} = \frac{RL_S L}{RLL_S}$$

(23)

$$R_T = R + RLL_S + RL + RLL_S$$

(24)

$$L_{ST} = L_S + RLL_S + RLL_S$$

(25)

$$L_T = L + R + RLL_S$$

(26)

RLL$_S$ denotes the concentration of the isostoichiometric ternary complex of the labeled ligand, unlabeled ligand, and receptor. $K_{D3}$ and $K_{D4}$ are dissociation constants. For thermodynamic reasons, the model only permits three independent dissociation constants. We can thus express $K_{D4}$ as a function of the other three (eq 23). Solving the eight equations for $L_T$ while eliminating $R$, $L_S$, $RL_S$, $L$, $RL$, and $RLL_S$ yields eq 27 ($K_{D3} \neq K_{D1}$).

$$L_T = \frac{j([l - (1 - F_{SB})K_{D2}K_{D3}]K_{D1} + (1 - F_{SB})K_{D2}K_{D3}^2)}{k(1 - F_{SB})(K_{D1} - K_{D3})K_{D1}}$$

(27)

with

$$j = L_{ST}F_{SB}^2 - (K_{D1} + L_{ST} + R_T)F_{SB} + R_T$$

$$k = L_{ST}F_{SB}^2 - (K_{D3} + L_{ST} + R_T)F_{SB} + R_T$$

and

$$l = L_{ST}F_{SB} - K_{D3} - L_{ST}$$

We note that eq 27 is a fifth-degree polynomial in $F_{SB}$, and therefore, an explicit solution for $F_{SB}$ cannot generally be given. Nevertheless, numerical methods allow efficient fitting of experimental data to eq 27 (31). We shall note that $F_{SB}$ has a bounded limit for $L_T \to \infty$, as given by eq 28.

$$\lim_{L_T \to \infty} F_{SB} = \frac{K_{D1} + L_{ST} + R_T - \sqrt{(K_{D3} + L_{ST} + R_T)^2 - 4L_{ST}R_T}}{2L_{ST}}$$

(28)

Depending on the value of $K_{D3}$, this limit ranges from 0 to 1 and reflects asymptotically incomplete displacement. In section 2.3 (Figure 3B, vide infra), the dependence of $F_{SB}$ on $L_T$, $K_{D2}$, and $K_{D3}$ is discussed and graphically illustrated by numerical simulations.

We can model nonspecific binding effects of the unlabeled ligand by modifying eq 26 to give eq 29.

$$L_T = L + R + RLL_S + NL$$

(29)

Solving for $L_T$ ($K_{D3} \neq K_{D1}$), we find eq 30, which is identical to eq 27, except that $K_{D2}$ is replaced everywhere by $(1 + N)K_{D2}$.

$$L_T = \frac{j([l - (1 - F_{SB})(1 + N)K_{D2}K_{D3}]K_{D1} + (1 - F_{SB})(1 + N)K_{D2}K_{D3}^2) + L_{ST}F_{SB} - K_{D3} - L_{ST}}{k(1 - F_{SB})(K_{D1} - K_{D3})K_{D1}} - \sqrt{(K_{D3} + L_{ST} + R_T)^2 - 4L_{ST}R_T}}{2L_{ST}}$$

(30)

Just like in the complete competitive binding model, we see that it is not possible to separate specific from nonspecific effects based on competitive experiments alone. Equations 19, 21, and 30 can be useful, if an estimate of $N$ has been obtained independently, e.g., from a direct binding experiment (see section 1.1). Alternatively, if nonspecific effects are neglected, $K_{D2}$ always provides a robust upper bound estimate of the true dissociation constant.

From simulations (see section 2.3), we find that, if $K_{D3} > K_{D1}$, we observe displacement of bound $L_S$ (i.e., decrease in $F_{SB}$) for an increase in $L_T$ (anticooperativity). Conversely, if $K_{D3} < K_{D1}$, we observe an increase in bound $L_S$ (i.e., increase in $F_{SB}$) for an increase in $L_T$ (cooperativity). In both cases, $F_{SB}$ converges to a precise, bounded limit (eq 28). Let us finally consider the special case where $K_{D3} = K_{D1}$. Under this condition, the system of eqs 1, 4, 13, 22–25, and 29 can be solved explicitly for $F_{SB}$, while eliminating $R$, $L_S$, $RL_S$, $L$, $RL$, and $RLL_S$. The result is identical to eq 6. Since eq 6 is independent of $L_T$, we find that $F_{SB}$ remains constant, independent of the amount of unlabeled ligand present. This is because, under these particular conditions, labeled and unlabeled ligands do not influence each other’s binding equilibria with the receptor.

1.4. Relationships between FP Observables and Binding States. When a fluorophore is excited with polarized light, the emitted light is also polarized as a function of fluorescence lifetime and rotational diffusion. The corresponding states.

When a fluorophore is excited with polarized light, the emitted light is also polarized as a function of fluorescence lifetime and rotational diffusion. The corresponding FP observables are typically the fluorescence intensities parallel, $I^\parallel$, and perpendicular, $I^\perp$, to the plane of exciting light. We assume in the following that the relative sensitivities of the two acquisition channels have been calibrated, a procedure commonly termed G factor correction (21). The values for polarization, $P$, and anisotropy, $A$, are then defined by eqs 31 and 32.

$$P = \frac{I^\perp - I^\parallel}{I^\parallel + I^\perp}$$

(31)

$$A = \frac{I^\perp - I^\parallel}{I^\parallel + 2I^\perp}$$

(32)

Both $P$ and $A$ are in principle unitless, but polarization is sometimes given in the literature as 1000 $P$ in units of mP (“millipolarization”). We will see that for algebraic simplicity it is preferable to work with anisotropy. The two parameters
are related by the nonlinear relationship given in eq 33.

\[ A = \frac{2P}{3 - P} \]  

(33)

The anisotropy of a rigid system that is not subject to rotational diffusion is called the limiting anisotropy, \( A_0 \), and is given by eq 34, where \( \kappa \) is the angle between absorption and emission transition dipoles (33).

\[ A_0 = \frac{3 \cos^2 \kappa - 1}{5} \]  

(34)

If the fluorescent probe retains residual flexibility with respect to a covalently tethered, completely immobilized binding epitope (e.g., a peptide), \( A_0 \) can be approximated by eq 35, where \( \lambda \) represents the angle associated with local motion (22).

\[ A_0 = \left( \frac{3 \cos^2 \kappa - 1}{5} \right) \left( \frac{3 \cos^2 \lambda - 1}{2} \right) \]  

(35)

It can be seen that the maximum value of \( A_0 \) is 0.4. \( A \) is related to \( A_0 \), the fluorescence lifetime of the fluorophore, \( \tau_F \), and the rotational correlation time of the observed molecule, \( \tau_C \), according to eq 36.

\[ A = \left(1 + \frac{\tau_F}{\tau_C}\right)^{-1} \times A_0 \]  

(36)

where \( \tau_F \) is characteristic for the type of fluorophore used, e.g., ~4 ns for fluorescein-based labels. \( \tau_C \) can be estimated from eq 37, if we assume first-order spherical solution geometry of the labeled species.

\[ \tau_C = \frac{\eta M V_{HS}}{R T} \approx (4.1 \times 10^{-13} \times M) s Da^{-1} \]  

(37)

For this estimate at 293.0 K, the aqueous viscosity, \( \eta \), is 0.010 g cm\(^{-1}\) s\(^{-1}\) (assumed to be solute-independent for dilute solutions), the hydrated specific volume, \( V_{HS} \), is typically 1.0 cm\(^3\)/g for proteins, and the universal gas constant, \( R \), is 8.314 J K\(^{-1}\) mol\(^{-1}\). \( M \) denotes the molecular mass of the labeled species in Daltons. Sophisticated corrections for other geometries and more general estimation approaches have been developed (31, 34, 35).

Multicomponent mixtures are described by eq 38, where \( A_{\text{OBS}} \) is the total observed anisotropy, \( \phi_m \) is the fractional fluorescence intensity (or quantum yield) of the \( m \)th component, and \( A_m \) is the anisotropy of the \( m \)th component.

\[ A_{\text{OBS}} = \sum_m \phi_m A_m \]  

(38)

with

\[ \sum_m \phi_m = 1 \]

The principal reason for the preferential use of \( A \) over \( P \) for quantitative analyses is that this simple linear superposition principle does not hold for \( P \). It should be noted here that the literature is rich in examples documenting confusion over this fact (23, 24, 36–38).

FIGURE 4: (A) Influence of \( Q \) on the observed anisotropy in the direct binding model (eqs 9 and 39). (B) Influence of \( Q \) on the observed anisotropy in the complete competitive binding model (eqs 17 and 39).

In the particular case of a mixture of two distinct species, e.g., free and bound labeled ligand (such as in direct or competitive FP binding experiments), \( A_{\text{OBS}} \) can be expressed as eq 39.

\[ A_{\text{OBS}} = \frac{Q F_{\text{SB}} A_B + (1 - F_{\text{SB}}) A_F}{1 - (1 - Q) F_{\text{SB}}} \]  

(39)

\( A_B \) and \( A_F \) denote anisotropies of bound and free species, respectively. \( Q \) is the ratio of fluorescence intensities (or quantum yields) of bound and free species measured under the same experimental conditions. In practice, \( Q \) is often found to be very close to 1, if the fluorophore is not directly involved in the binding event (31). A \( Q \) of <1 would, for example, mean that quenching of the fluorophore occurs upon binding. Solving eq 39 for \( F_{\text{SB}} \), we find eq 40.

\[ F_{\text{SB}} = \frac{A_{\text{OBS}} - A_F}{(A_B - A_{\text{OBS}})Q + A_{\text{OBS}} - A_F} \]  

(40)

Equations 39 and 40 provide a direct link between the experimental observables in FP and the theoretical description of direct and competitive binding as explored in sections 1.1–1.3. Graphical illustrations of these relationships are provided in section 2.4 (Figure 4, vide infra).

1.5. Sensitivity of Competitive Binding Assays. The sensitivity, \( S \), of a competitive binding assay can be defined as the change in the fraction of the bound labeled ligand (\( F_{\text{SB}} \)) relative to the change of the total unlabeled competitor (\( L_T \)), as shown in eq 41.
\[
S = \left| \frac{dF_{SB}}{dT} \right|
\]  

(41)

where \( S \) is a function of \( K_{D1}, K_{D2}, L_{ST}, L_{T}, \) and \( R_{T} \). We seek to identify sets of initial conditions defined by these parameters that maximize \( S \). Thus, knowledge of the functional dependence of \( S \) on these parameters is crucial for designing and implementing optimal FP screening assays.

For the following considerations, we take the functional forms for \( L_{T} \) and \( F_{SB} \) from eqs 16 and 17, respectively. We define a function \( f(L_{T}) \) according to the right side of eq 17 such that \( F_{SB} = f(L_{T}) \). We note that \( 0 < f(L_{T}) < 1 \) and that \( f(L_{T}) \) is continuous and monotonically decreasing. For the inverse of \( f, f^{-1} \), we find consequently that \( L_{T} = f^{-1}(F_{SB}) \), where \( f^{-1}(F_{SB}) \) is given by the right side of eq 16. The total derivatives of \( f \) and \( f^{-1} \) with respect to their arguments are related by the standard relation of eq 42.

\[
\frac{d}{dL_{T}}[f(L_{T})] = \left\{ \frac{d}{dF_{SB}}[f^{-1}(F_{SB})] \right\}^{-1}
\]  

(42)

Both derivatives are negative. Hence, we seek a minimum of the derivative of \( f \) or, equivalently, a maximum of the derivative of \( f^{-1} \). Since the left side of eq 42 is rather cumbersome to evaluate explicitly, we can make beneficial use of the right side of this relationship. We first solve eq 16 for \( R_{T} \) to obtain eq 43.

\[
R_{T} = \frac{n[(1 - F_{SB})L_{ST} + K_{D1}] + (1 - F_{SB})K_{D1}L_{T}F_{SB}}{n(1 - F_{SB})}
\]  

(43)

with

\[
n = (K_{D1} - K_{D2})F_{SB} + K_{D2}
\]

Differentiating \( f^{-1} \) with respect to \( F_{SB} \) and then substituting \( R_{T} \) with the right side of eq 43 yields eq 44.

\[
\frac{d}{dF_{SB}}[f^{-1}(F_{SB})] = -\frac{n^{2}(1 - F_{SB})^{2}L_{ST} + K_{D1}] + (1 - F_{SB})^{2}K_{D1}K_{D2}L_{T}}{n(1 - F_{SB})^{2}F_{SB}K_{D1}}
\]  

(44)

A necessary condition for a maximum of \( (d/dF_{SB})[f^{-1}(F_{SB})] \) is then given by eq 45.

\[
\frac{d^{2}}{dF_{SB}^{2}}[f^{-1}(F_{SB})] = 0
\]  

(45)

Direct computation leads to eq 46 and provides the desired optimality criterion for \( K_{D1}, K_{D2}, L_{ST}, L_{T}, F_{SB}, \) and thence \( R_{T} \) via eq 43.

\[
[(K_{D1} - K_{D2})F_{SB} + K_{D2}]^{2}[(2(K_{D1} - K_{D2})F_{SB}^{2} + 3K_{D2}F_{SB} - K_{D1}(1 - F_{SB})^{2}K_{D2}L_{ST}) - (1 - F_{SB})^{3}[2(K_{D1} - K_{D2})F_{SB} + K_{D2}]K_{D1}K_{D2}L_{T}] = 0
\]  

(46)

Equation 46 is a fifth-degree polynomial in \( F_{SB} \). In practice, \( K_{D1} \) and \( L_{ST} \) are given by the assay specifications, and \( L_{T} \) is set to zero to evaluate the initial competitive sensitivity. Optimal values of \( F_{SB} \) (and \( R_{T} \)) are then obtained by numerically solving eq 46 for those values of \( K_{D2} \), for which assay sensitivity is to be maximized (31). The advantage of this procedure is that a competitive assay may be a priori optimized for a desired target range of expected competitor dissociation constants. Section 2.5 illustrates the above equations by numerical simulations based on conditions that are typically encountered in practice (Figure 5, vide infra).

1.6. **Assay Quality, Data Analysis, and Ranking Strategies**

FP assay quality can be assessed by two parameters, signal-to-noise ratio (S/N) and \( Z' \) factor, as given by eqs 47 and 48 (39).

\[
S/N = \frac{A_{U} - A_{F}}{\sqrt{U^{2} + \sigma_{U}^{2}}}
\]  

(47)

\[
Z' = \frac{(A_{U} - A_{F}) - (3\sigma_{U} + 3\sigma_{F})}{A_{U} - A_{F}}
\]  

(48)

where \( A_{U} \) and \( A_{F} \) denote the upper (i.e., at a given \( R_{T} \)) and lower/free (i.e., \( R_{T} = 0 \)) mean anisotropy bounds under specific assay conditions (i.e., at given \( L_{ST} \) and \( K_{D1} \)) with associated standard deviations, \( \sigma_{U} \) and \( \sigma_{F} \), respectively. The
Z' factor describes the normalized gap size between the three-
standard deviation bands around average upper and lower
anisotropies. Assays wherein Z' > 0.5 are considered
excellent (39), as illustrated in the subsequent paper (31).

The primary goal of initial data analysis is to eliminate
FP data from intrinsically fluorescent or quenching com-
petitors. Since the value of \( I^* + 2I^I \) is directly propor-
tional to total fluorescence intensity of the sample, this can be
achieved by statistically analyzing the distribution and
removing potential outliers (18).

Subsequently, the results from a high-throughput screen
need to be ranked numerically for the identification of the
most potent and promising hits. To this end, we introduce
four ranking functions, \( R_1 - R_4 \). \( A_{OBS} \) denotes in the following
the observed assay anisotropy for a specific competitor \( L \).
A_1 is a linearized change of anisotropy model defined by eq
49.

\[
R_1 = \frac{A_{OBS} - A_U}{A_U - A_F} \times 100\%
\]

(49)

\( R_1 \) is a \( \sigma \) deviation model based on eq 50.

\[
R_2 = \frac{A_{OBS} - A_U}{\sigma_U}
\]

(50)

\( R_2 \) provides a single-point estimate of the dissociation
constant of the competitor-receptor complex, \( K_{D2} \), as given
by eq 51. As shown in the Supporting Information, this
estimate is obtained by solving eqs 1, 3, 4, and 13−15 for
\( K_{D2} \) and expressing \( F_{SB} \) in terms of known quantities via eqs
6 and 40. \( R_3 \) is likely the most robust parameter, since it
takes actual compound screening concentrations into
account, and may allow comparisons even across different
types of assays.

\[
R_3 = \frac{-L_{ST}F_{SB}^2 + (K_{D1} + L_{ST} - L_T + R_T)F_{SB} + L_T - R_T} {(1 - F_{SB})(L_{ST}F_{SB}^2 - (K_{D1} + L_{ST} + R_T)F_{SB} + R_T)}
\]

(51)

with

\[
F_{SB} = [2(A_{OBS} - A_F)R_T]/
\left\{ [K_{D1} + L_{ST} + (1 - 2Q)R_T + \sqrt{\sigma}]A_U - (K_{D1} + L_{ST} + R_T + \sqrt{\sigma})A_F + 2(1 - Q)R_T A_{OBS} \right\}
\]

and

\[
\sigma = (K_{D1} + L_{ST} + R_T)^2 - 4L_{ST}R_T
\]

which, when \( Q = 1 \), simplifies to

\[
F_{SB} = \frac{A_{OBS} - A_F}{A_U - A_F} \left( \frac{K_{D1} + L_{ST} + R_T - \sqrt{\sigma}}{2L_{ST}} \right)
\]

(52)

\( R_4 \) is a total fluorescence change model according to eq 52.

\[
R_4 = \frac{I_{OBS} - I_{EXP}}{I_{EXP}} \times 100\%
\]

(52)

with

\[
I_{EXP} = \frac{[1 - (1 - Q)F_{SB}]I_M}{1 - (1 - Q)p}
\]

and

\[
p = \frac{K_{D1} + L_{ST} + R_T - \sqrt{(K_{D1} + L_{ST} + R_T)^2 - 4L_{ST}R_T}}{2L_{ST}}
\]

which, when \( Q = 1 \), simplifies to

\[
R_4 = \frac{I_{OBS} - I_M}{I_M} \times 100\%
\]

(52)

\( I_{OBS} \) denotes the observed value of \( I^* + 2I^I \) for a specific competitor \( L \). \( I_{EXP} \) is the expected, \( Q \)-corrected value at a
given \( F_{SB} \), and \( I_M \) is the mean assay value of \( I^* + 2I^I \) without
competitors. \( F_{SB} \) is to be taken from the expression in eq
51. \( R_1 \) is a useful control parameter for assessing whether
competitors that score well in \( R_1 - R_3 \) systematically influence
the total fluorescence intensity (31).

2. SIMULATIONS

In the second part of this paper, we will visually illustrate
the key results obtained in the theoretical part. We will use
physically realistic ranges for all parameters as they typically
apply to FP assays. In all cases, \( L_{ST} \) was set to 30 nM (31).
In general, \( L_{ST} \) needs to be much smaller than \( R_T \), because
otherwise excess amounts of free ligand would always be
present, even without any added competitor. On the other
hand, \( L_{ST} \) needs to be large enough to be detected at a good
signal-to-noise ratio in the linear range of the fluorescence
polarimeter (18, 31). Simulations were carried out with
Mathematica 4.1 (Wolfram Research, Champaign, IL) and
SigmaPlot 2001 (SPSS, Chicago, IL).

2.1. Direct Binding. Characteristic, semilogarithmic direct
binding curves without nonspecific effects (\( N_S = 0 \)) are shown
in Figure 2A. \( F_{SB} \) is depicted as a function of \( R_T \) for values of \( K_{D1} \)
ranging from 0.01 to 1000 \( \mu \)M. Equation 9 was used. Nonspecific effects are illustrated in Figure 2B.
\( K_{D1} \) was set to 1 \( \mu \)M, and binding curves are shown for \( N_S \)
values ranging from 0 to 0.6. The axis on the right indicates the
values of \( F_{SBA} \) (i.e., using the false assumption \( N_S = 0 \)) for
the blue curve, for which the true \( N_S \) is equal to 0.6. The
corresponding value of \( K_{D1A} \) for the blue curve is 1.6 \( \mu \)M.
Equations 9, 11, and 12 were used.

2.2. Complete Competitive Binding. Characteristic complete
competitive binding curves without nonspecific effects
\( (N_S = 0) \) are shown in Figure 3A. With both \( R_T \) and \( K_{D1} \) set
equal to 1 \( \mu \)M, curves for values of \( K_{D2} \) ranging from 0.001
to 1000 \( \mu \)M are depicted. Equation 17 was used. Note that
with an increasing affinity of the competitor (i.e., decreasing
\( K_{D2} \)) \( F_{SB} \) approaches zero when \( L_T \approx R_T \), i.e., when total
competitor and receptor concentrations are approximately
equal. It is evident that accurate data fitting and estimation
of \( K_{D2} \) become increasingly difficult for lower values of \( K_{D2} \),
since competition curves become more and more closely
spaced. One way to address this limitation is to repeat the
measurement at lower concentrations of \( R_T \), which renders
the assay more discriminatory for smaller values of \( K_{D2} \).
Lowering $R_T$, however, concomitantly reduces the observed anisotropy in the absence of competitor, $A_{OB}$, and thus the assay’s signal-to-noise ratio. In practice, competitive binding of high-affinity ligands is best studied at the lowest $R_T$ permitted by signal-to-noise considerations.

2.3. Incomplete Competitive Binding. Characteristic incomplete competitive binding curves without nonspecific effects ($N = 0$) are shown in Figure 3B. $R_T$ and $K_{D1}$ were set equal to 1 μM, $K_{D2}$ equals 1 μM (red curves) or ranges from 0.1 to 10 μM (blue curves). $K_{D3}$ ranges from 0.01 to 1000 μM. Equation 27 was used. The graphs illustrate the effects of the three independent dissociation constants, $K_{D1} - K_{D3}$. $K_{D1}$ determines the value of $F_{SB}$ when $L_T = 0$; $K_{D2}$ shifts transition curves horizontally with respect to $L_T$, and $K_{D3}$ specifies the asymptotic value of $F_{SB}$ for large $L_T$ values and, thereby, the degree of cooperativity or anticooperativity. When $K_{D2} = K_{D3}$, $F_{SB}$ remains constant and independent of $L_T$. The asymptotic limits of $F_{SB}$ for $L_T \rightarrow \infty$ depend on $K_{D3}$ and are given by eq 28.

2.4. Relationship between FP Observables and Binding States. Figure 4A illustrates the effects of fluorescence intensity differences between bound and free labeled ligand (ratio expressed by $Q$) on observable anisotropy, $A_{OBS}$, in the direct binding model. For generality, $A_{OBS}$ is shown normalized with respect to the difference between bound and free anisotropies, $A_{B} - A_{F}$. $K_{D1}$ and $N_S$ were set equal to 1 μM and 0, respectively. $Q$ ranges from 0.1 to 10. Equations 9 and 39 were used. If it were (falsely) assumed for the blue curves that $Q = 1$, $K_{D1}$ would be misestimated as 10.0200 ± 0.0014 μM ($Q = 0.1$, i.e., quenched fluorescence) and 0.0881 ± 0.0003 μM ($Q = 10$, i.e., enhanced fluorescence) by a nonlinear least-squares fit.

Figure 4B depicts the influence of $Q$ on $A_{OBS}$ for the case of complete competitive binding. $R_T$, $K_{D1}$, and $K_{D2}$ were set to 1 μM ($N = 0$). $Q$ ranges from 0.1 to 10. Equations 17 and 39 were used. If it were (falsely) assumed for the blue curves that $Q = 1$, one would obtain the following: $K_{D1} = 10.1300 \pm 0.0002$ μM and $K_{D2} = 1.0080 \pm 0.0002$ μM ($Q = 0.1$), or $K_{D1} = 0.0983 \pm 0.0001$ μM and $K_{D2} = 0.9947 \pm 0.0006$ μM ($Q = 10$) by a nonlinear least-squares fit. The noteworthy result is that, despite misestimation of $K_{D1}$, a correct estimation of $K_{D2}$ is very insensitive to large errors in the estimate of $Q$. This finding is of great practical interest, since the ultimate goal of competitive binding assays is precisely the accurate characterization of competitor binding ($K_{D2}$), whereas the accuracy of the dissociation constant of the labeled probe ($K_{D1}$) is less significant.

2.5. Sensitivity of Competitive Binding Assays. Sensitivity-optimized values of $F_{SB}$ at $L_T = 0$ are shown in Figure 5A as a function of $K_{D2}$. $K_{D1}$ ranges from 0.1 to 10 μM. Equation 46 was used. The corresponding optimal values of $R_T$ can be calculated from eq 43. For example, in our FP assay for inhibitors of the VIVIT–calcineurin interaction ($K_{D1} = 0.5$ μM, $I_{50T} = 30$ nM) (31), calcineurin concentrations of 0.52, 0.33, and 0.11 μM deliver optimal sensitivity for identifying small-molecule inhibitors with projected $K_{D2}$ values of 100, 1, and 0.05 μM, respectively. The corresponding bound fractions of labeled VIVIT ligand ($F_{SB}$) are 0.50, 0.39, and 0.17, respectively. It is clear that the optimal sensitivity for high-affinity competitors is usually achieved when $F_{SB} < 0.5$.

Figure 5B depicts a two-dimensional contour plot, in which the normalized assay sensitivity, $S_{MAX}$, at $L_T = 0$ is graphed as a function of both $F_{SB}$ and $K_{D2}$ ($K_{D1} = 1$ μM). Equations 41 and 44 were used. Choices of parameter pairs that fall into the red or orange zones are favorable.

3. SUMMARY AND PROCEDURAL FLOWCHART

This paper develops a general theoretical framework for equilibrium binding experiments. Our only prior assumption was that thermodynamic ideality applies such that thermodynamic activities and concentrations are equivalent (40). All relationships are conveniently expressed in terms of a priori known total input concentrations. Exact algebraic relationships were derived for the determination of dissociation constants in competitive binding experiments.

A four-state model was introduced that can account for the frequently observed phenomenon of incomplete competitive dissociation. We showed that the neglect of potential nonspecific binding effects will, at worst, lead to overestimation of the respective dissociation constant, i.e., introduce a
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conservative error corresponding to an upper bound estimate. Furthermore, we demonstrated that, even when the ratio \( Q \) of bound and free labeled ligand quantum yields is severely misestimated, an accurate estimation of the dissociation constant of the unlabeled competitor can nevertheless be obtained. These findings rationalize the great robustness of the competitive binding experimental design. Our sensitivity analysis provides guidance for the optimal choice of experimental parameters.

As a specific application, the theoretical framework was then linked to experimental observables as they occur in FP. Finally, scoring and ranking strategies were developed for application in high-throughput FP assays. Figure 6 lays out a procedural flowchart that summarizes the modular core elements of a competitive high-throughput FP screening effort, using the concepts developed in this paper. We shall point out that a very critical point for a successful screen is the careful choice of a labeled ligand. Its binding affinity for the receptor should be high, and the fluorophore should not be influenced by or interfere with the binding state; the fluorescence lifetime of the fluorophore needs to be appropriate, and nonspecific binding should be minimal. The subsequent paper (31) describes as a detailed practical example and application our experiences with the screening for small-molecule inhibitors of the protein—protein interaction between human calcineurin and NFAT (31).

SUPPORTING INFORMATION AVAILABLE

Derivation of key equations illustrated with Mathematica 4.1 (Wolfram Research, Champaign, IL). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

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