Chapter 6

Application of Fluorescence Polarization in HTS Assays

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Abstract

Steady-state measurements of fluorescence polarization have been widely adopted in the field of high-throughput screening for the study of biomolecular interactions. This chapter reviews the basic theory of fluorescence polarization, the underlying principle for using fluorescence polarization to study interactions between small-molecule fluorophores and macromolecular targets, and representative applications of fluorescence polarization in high-throughput screening.

Key words: FP, Polarization, Anisotropy, Competition binding, High-throughput screening.

1. Introduction

Fluorescence polarization (FP) is a powerful fluorescence-based technique for the study of biomolecular interactions in aqueous solution. For a small-molecule fluorophore or a small molecule labeled with a fluorescent moiety, the interaction with a macromolecule can be monitored through the increase in FP, which occurs with the change in fluorophore mobility upon complex formation. Perrin first described the quantitative relationship of the observed polarization with molecular size and solution viscosity in 1926 *(1)*, and Weber subsequently applied FP to biological systems [for a review see *(2)*]. FP has since been applied to a wide range of interactions including DNA–DNA interactions, DNA–protein interactions, protein–protein interactions, and small molecule–protein interactions *(3–7)*. Migration of high-throughput screening (HTS) in the biopharmaceutical industry to fluorescence and luminescence formats and development of commercial microplate FP instruments in the mid-1990s have resulted in the explosive growth of FP applications in HTS over the last decade.

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1.1. Fluorescence Polarization Basic Theory

Fluorescence polarization, or anisotropy, is a property of fluorescent molecules that can be measured using a FP instrument. A polarized light is utilized to excite a fluorescent sample and the emission light intensities of the channels that are parallel (I_S) and perpendicular (I_P) to the electric vector of polarized excitation light are collected. The difference, $I_S - I_P$, can be normalized by the total fluorescence intensity of the emission beam, $I_S + I_P$ (polarization), or by the total fluorescence emission intensity from the sample, $I_S + 2I_P$ (anisotropy). Polarization (P) is then defined as $(I_S - I_P)/(I_S + I_P)$ and anisotropy (A) is defined as $(I_S - I_P)/(I_S + 2I_P)$. Both polarization (P) and anisotropy (A) terms have been widely used. Polarization and anisotropy can be interconverted by the equation $A = 2P/(3 - P)$. Anisotropy is preferred for analyzing complex systems because the equations are considerably simpler when expressed in this term *(8)*. Since polarization (P) is nearly linearly correlated with anisotropy (A) because of a limiting anisotropy value of 0.4 (*vide infra*; **Fig. 6.1**), applications expressed in polarization are still valid in practical terms.

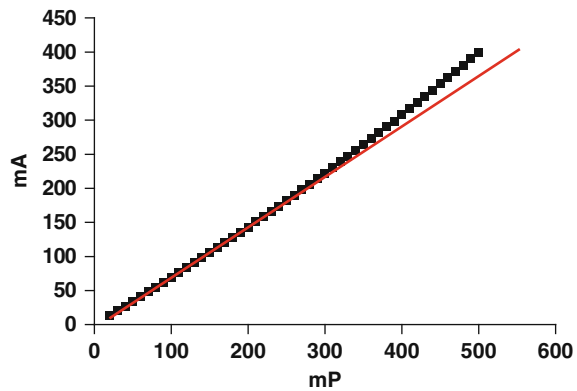

Fig. 6.1. The relationship between polarization and anisotropy. The data are generated by the equation $A = 2P/(3 - P)$. The thin, straight line depicts theoretic perfect linear correlation.

In order to more fully comprehend FP, one needs to start with the absorption of excitation light by a fluorophore. When a fluorescent sample is illuminated by polarized light, those molecules with their absorption transition dipole aligned parallel to the electric vector of the polarized excitation have the highest probability of absorption, resulting in polarized emission light that is also parallel to the polarized excitation. Had all molecules in this sample been fully aligned parallel to excitation, the sample would have had anisotropy of 1. In reality, fluorescent molecules in solution are completely randomized relative to excitation. The probability of absorption is proportionally dependent on the angle between the fluorophore absorption dipole and the

polarized excitation. This photoselection process results in polarized emission with a theoretical maximum anisotropy of 0.4 *(8)* (*see* **Note 1**). The observed anisotropy of a given sample falls between 0 and 0.4, depending on many extrinsic factors at play during the fluorescence lifetime of the fluorophore. The primary determinant of fluorescence depolarization in dilute solutions is the rotational diffusion of the fluorophore *(1, 9, 10)*. For ideal spherical rotors, anisotropy measured under steady-state conditions follows the Perrin equation [*1*], where A_0 is the intrinsic anisotropy, τ is the fluorescence lifetime, and θ is the rotational correlation time of the fluorophore (the time the fluorophore rotates through an angle of 1 radian), which in turn is proportional to the viscosity of the solution (η) and the molecular volume of the rotor (V) and inversely proportional to the temperature (T) [*2*].

$$A = \frac{A_0}{1 + \tau/\theta} \quad [1]$$

$$\theta = \frac{\eta V}{RT} \quad [2]$$

The consequence of this in practical terms is that fluorescence anisotropy can be used to measure changes in the rotational diffusion rate of a fluorophore as illustrated in **Fig. 6.2**. As a result, FP measurements can yield information on the size and shape of the

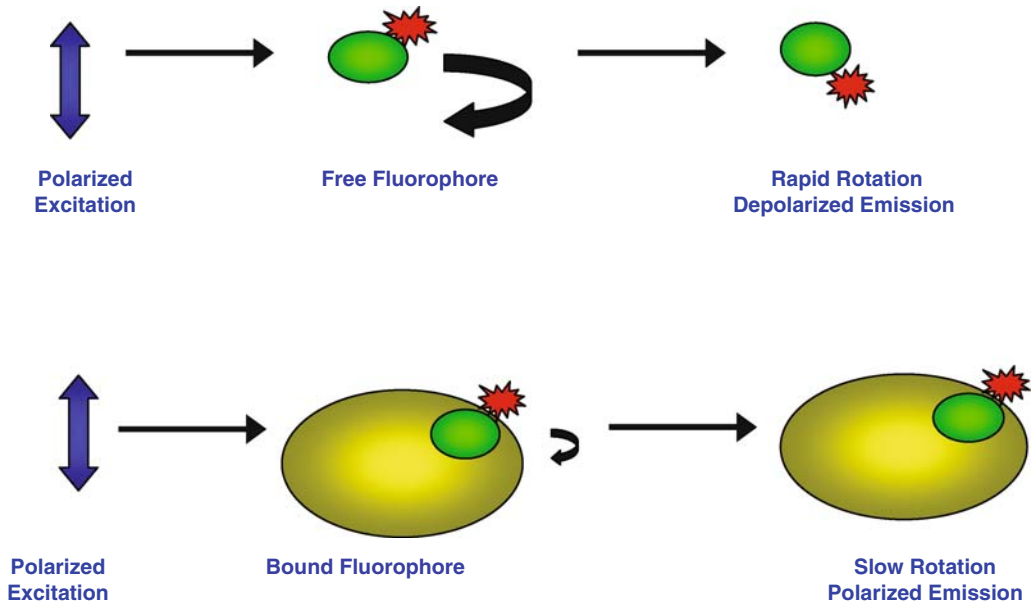

Fig. 6.2. The basics of fluorescence polarization-binding assays. The small-molecule fluorophores free or bound to macromolecules can be excited by vertically polarized light, resulting in polarized emission. The observed steady-state polarization of the sample depends on the extent of fluorophores bound to macromolecules. Free fluorophores have low observed polarization due to fast rotation relative to fluorescence lifetime, while bound fluorophores have high observed polarization due to slow rotation.

fluorophore and the molecule complexed with the fluorophore. In non-viscous aqueous solutions, a typical small-molecule fluorophore rotates on a timescale of 40 ps or less *(11)*, much faster than a typical fluorescence lifetime of 10 ns *(11)*, which results in depolarized emission. Upon fluorophore binding to a macromolecule, the complex will rotate much slower with a rotational correlation time on par with the timescale of typical fluorescence lifetime, resulting in polarized emission. This forms the basis for quantifying the fraction of the fluorophore bound to the macromolecule. **Figure 6.3** delineates the relationship between fluorescence anisotropy, carrier molecular weight, and fluorescence lifetime (simulated data using [*1*] assuming a limiting anisotropy of 0.4 and assuming the fluorophore is rigidly attached to a spherical carrier) *(12)*. It is evident that typical fluorophores such as fluorescein and BODIPY have ideal fluorescence lifetimes that allow FP measurements between a small labeled probe (<~1,500 Da) and a macromolecule receptor (>~15,000 Da).

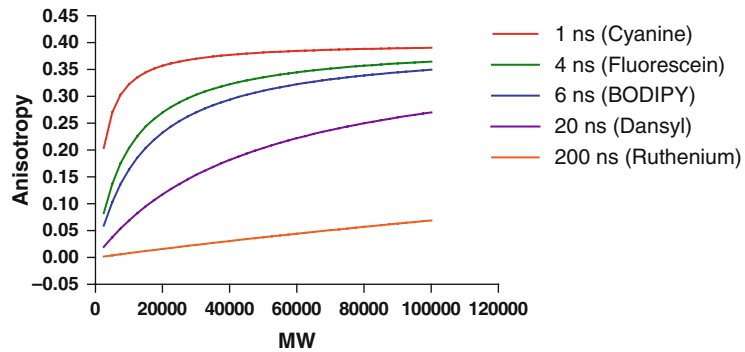

Fig. 6.3. Simulated graph illustrating the dependence of fluorescence anisotropy on fluorescence lifetime of the fluorophore and molecular size of the carrier macromolecule. Data are generated using [*1*].

2. Methods

Numerous FP applications have been developed for HTS based on the principles described above and shown in **Fig. 6.2**. Because fluorescence polarization is a ratiometric measurement, in theory the FP signal should have less interference from background fluorescence of the assay plate and buffer. Because of this advantage, FP has become a popular technique for HTS assays. FP can be determined by steady-state measurements or time-resolved measurements *(13)*. In the time-resolved FP measurement, a short

pulse of light excites the sample and the emission is recorded with a high-speed detection system that allows measurements on the nanosecond scale. In a steady-state FP measurement, the sample is illuminated by continuous excitation light and the measurement is an average of the time-resolved phenomenon over the intensity decay of the sample. The steady-state FP averaging over a single exponential decay may thus mask complex exponential delays in some systems and could be one of the reasons for some nonideal, steady-state FP data observed. The overwhelming majority of FP applications in HTS are steady-state measurements using commercial fluorescence plate readers. The scope of this chapter is limited only to steady-state FP measurements. This chapter will take a look at three steady-state FP applications in HTS: (i) direct FP competition-binding assay; (ii) FP used as a detection method in a functional assay; (iii) determination of binding mechanism from an FP competition-binding assay. Representative examples of the applications are presented where applicable.

2.1. Direct FP Competition Assay

The objective of a direct FP competition assay is to identify compounds that compete with the small-molecule fluorescent probe for binding to the macromolecular target. This approach is a quick and easy method that has been extensively used to identify active-site binders of enzyme targets and small-molecule binders of nuclear receptors (NR). The disadvantage is that the assay may not identify compounds that bind at sites remote from the fluorescent probe (**Section 2.3**). In addition to active-site binders, fluorescent probes prepared from allosteric ligands can be used to identify compounds that bind to regulatory sites outside of the target enzyme active site. In the following sections, the process for developing a FP competition assay for the exosite of a protease (FVIIa) and the utilization of a quality control parameter instead of an interference assay (counter-screen) in a direct nuclear receptor FP competition assay to identify false-positive hits are described.

2.1.1. Design and Synthesis of an Appropriate Fluorescent Probe

FP assay development begins with designing a fluorescent probe. There are no universal rules for how to design an ideal probe for FP. When a label is attached to a known ligand to prepare a probe, the probe will work in FP assays only when the following conditions are satisfied: (1) the attachment of the label does not significantly alter how the ligand binds to the target; (2) the label cannot have a strong propeller effect, i.e., the rotational diffusion motion of the label needs to be restricted upon the binding of the probe to the target. A routine practice in our lab is to design probes with various linkers (both type and length) and to experimentally determine if the probes have the expected potency in the activity assay relative to the unmodified ligand and if the probes work in the FP assay.

We applied the above approach to design a probe to identify compounds that bind at an allosteric site on the protease factor

VIIa (FVIIa) in complex with its cofactor, tissue factor (TF). TF/FVIIa is a well-validated anticoagulant target *(14, 15)*. E-76, Ac-ALCDDPRVDRWYCQFVEG-NH2 (disulfide bond), is a reported partial inhibitor of TF/VIIA amidolytic activity that binds to an exosite outside of the active site of FVIIa with a reported IC50 of 9.7 nM *(16)*. The mechanism of inhibition was confirmed in our lab, though an IC50 of 2.3 nM was obtained under our assay conditions. Next, the reported crystal structure of the FVIIa/E-76 complex was examined to determine the residues on E-76 that are solvent exposed and potential sites for probe attachment. Based on solvent accessibility in the three-dimensional structure, the Glu residue at the C-terminal end was mutated to a Lys residue for the covalent attachment of Hilyte Fluor 488. The designed probe retained the same interactions with FVIIa as E-76 in a computer model. The probe was then custom synthesized by Anaspec (San Jose, CA).

2.1.2. Determination of K_d Between the Fluorescent Probe and the Target

An appropriate concentration of the probe to use in the K_d determination is dependent upon several factors including the linearity of the fluorescent response with probe concentration, the quantum yield of the probe, the K_d between the probe and the target, and instrument sensitivity. The FVIIa experiments were carried out in an assay buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, and 0.005% (w/v) Triton X-100. Samples were prepared at a volume of 20 μl in a black 384-well low-volume polypropylene Matrical plate (cat# MP101-1-PP). Fluorescence intensity and anisotropy were measured on an Analyst AD plate reader (Molecular Devices). A probe dose titration was initially performed to determine the linearity of the fluorescent signal with probe concentration, and the probe fluorescence intensity was linear up to 500 nM. The probe concentration for the K_d determination should not be much greater than $2K_d$ to avoid stoichiometric titration *(17)*. A concentration of the probe corresponding to the IC50 in the functional assay is often chosen as the initial concentration for the binding assay. In this case, an initial probe concentration of 5 nM was selected, which yielded a fluorescence intensity S/B of 70.

Because there are intrinsic differences in instrument sensitivity for measuring the S channel and P channel, all plate readers need to be checked and calibrated for the instrument "G" or grating factor before obtaining any polarization data. The revised equation for polarization incorporating the G factor term is $P = (I_S - G \times I_P)/(I_S + G \times I_P)$. In this example, the G factor for the Analyst AD instrument was calibrated to achieve an mP of ~60 for the free probe (*see* **Note 2** for details on G factor calibration).

Next, a FVIIa concentration dependence was performed in the presence of 5 nM fixed probe concentration and 2000 nM sTF (**Fig. 6.4a**). Fitting the anisotropy data to equation [3a], where L_0

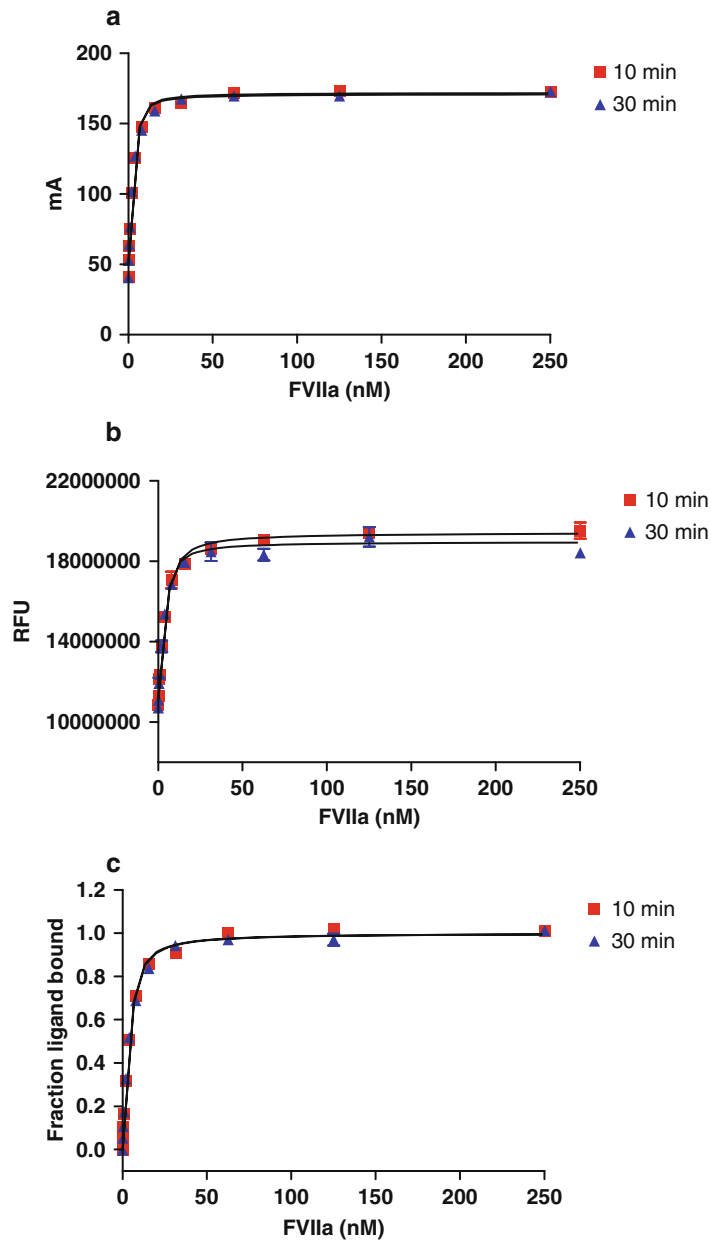

Fig. 6.4. The dose titration of FVIIa in the presence of fixed probe. The assay contained 5 nM probe, 2000 nM sTF, and various concentrations of FVIIa and was measured for anisotropy and fluorescence intensity on Analyst AD after 10 and 30 min incubation at room temperature. (**a**) Anisotropy measurement; (**b**) fluorescence measurement; (**c**) *fb* data converted from anisotropy data using [4].

is the total probe concentration, R_0 is the total enzyme concentration, a is the probe signal in the absence of ligand, and b is the probe signal in the presence of saturating concentrations of ligand, yields A1 (free probe), A2 (bound probe), and an approximate K_d

value of 0.6 nM. Identical results were observed after 10 min and 30 min incubation, indicating that the binding equilibrium was reached quickly. The same dose titration was also measured by fluorescence intensity. Fitting of the fluorescence intensity data to equation [3a] yielded a K_d of 1.3–1.8 nM (**Fig. 6.4b**). The discrepancy in K_d values obtained from anisotropy and fluorescence intensity measurements is attributed to the change in quantum yield of the probe upon binding to FVIIa. The quantum yield ratio (Q) of the bound probe to the free probe can be calculated from the ratio of fluorescence intensity of the bound probe to the fluorescence intensity of the free probe. A Q value of 1.78 was obtained from the fluorescence data in **Fig. 6.4b**. The FP data in **Fig. 6.4a** were converted to the fraction bound probe (fb) by equation [4]. Fitting of the FP fb data to equation [3b] yielded a K_d of 1.5–1.6 nM (**Fig. 6.4c**), which now agrees with the K_d calculated from the fluorescence intensity measurement. In addition, the selected probe concentration of 5 nM satisfies the non-stoichiometric conditions (i.e., the probe concentration not much greater than $2K_d$). Had the probe concentration been much greater than two times the calculated K_d value, a lower probe concentration would need to be selected and the K_d measurement repeated.

$$y = a + (b-a)\frac{(Kd + R_0 + L_0) - \sqrt{(Kd + R_0 + L_0)^2 - 4R_0L_0}}{2L_0} \quad [3a]$$

$$y = \frac{(Kd + R_0 + L_0) - \sqrt{(Kd + R_0 + L_0)^2 - 4R_0L_0}}{2L_0} \quad [3b]$$

$$fb = \frac{(A - A_1)}{(A - A_1) + Q(A_2 - A)} \quad [4]$$

Parallel with the determination of K_d between the fluorescent probe and the target, additional HTS assay optimization should be performed. As mentioned above, FP is a ratiometric method and background fluorescence effects are expected to be minimal. However, in practice a number of experimental details including the chemical and physical properties of the test compounds can introduce artifacts into the results.

Some plate types yield significantly larger assay windows for a given FP assay. Buffer optimization including detergent and carrier protein screening should be performed to select assay conditions that minimally affect the aggregation state of the probe and maximally enhance the stability of the target protein. For DMSO tolerance, it is critical that not only the assay signal window is maintained but also the interactions between the probe and the

target protein are not adversely affected. The order of reagent addition and the equilibration time need to be evaluated and optimized for FP competition assays that do exhibit time-dependent binding between the probe and the target.

When multiple fluorescent probes are available, the most potent probe with a reasonable fluorescence quantum yield should be selected for the FP competition assay. In general, the most potent probe affords the best chance to identify compounds with the widest potency range *(17)*.

2.1.3. Selection of Appropriate Screen Conditions for Direct FP Competition Assay

An *fb* of 0.5–0.8 is recommended for direct FP competition assays, which is a compromise between achieving a reasonable anisotropy signal window and adequate sensitivity to detect small-molecule compounds *(17)*. An *fb* value higher than 0.8 will decrease the assay sensitivity to identify weakly active compounds, while an *fb* value less than 0.5 often leads to an assay with an inadequate anisotropy signal window.

Based on data in **Fig. 6.4c**, the FVIIa concentration corresponding to an *fb* of 0.8 was selected for the TF/FVIIa FP competition assay. The optimized competition screen contains 5 nM probe and 12.5 nM FVIIa. The FP measurements are taken after 20 min incubation at room temperature. Upon finalization of the screen conditions, reagent working stock stability is then determined to ensure that the screen is conducted within the time constraints of reagent stability.

2.1.4. Validation of Direct FP Competition Assay

Validation of the FP competition assay can be achieved by the use of a nonfluorescent counterpart of the probe or a known positive control that binds at the same site as the probe. In this case, we performed the dose titrations with E-76 (**Fig. 6.5a**) as a positive control and compound 1, which binds to the FVIIa active site, as a negative control (**Fig. 6.5b**).

E-76 is fully competitive with the probe, consistent with E-76 and the probe binding to the same exosite on FVIIa. The IC50 of E-76 in the FP competition assay (23 nM) is several fold higher than the IC50 in the activity assay (2.3–9.7 nM), which is typical for FP competition assays. A lower starting *fb* will lead to a FP-derived IC50 closer to the functional assay IC50 but with the disadvantage of a smaller FP assay window *(17)*.

Compound 1 showed a minor degree of displacement with the probe, consistent with compound 1 and the probe binding at distinct sites on the target *(18)* and also consistent with partial inhibition of TF/FVIIa amidolytic activity by E-76. In the single-dose screen, this direct FP competition assay would be unlikely to identify hits that bind to alternate sites, such as compound 1 in this example. In **Fig. 6.5a** and **b**, a control reaction was performed in

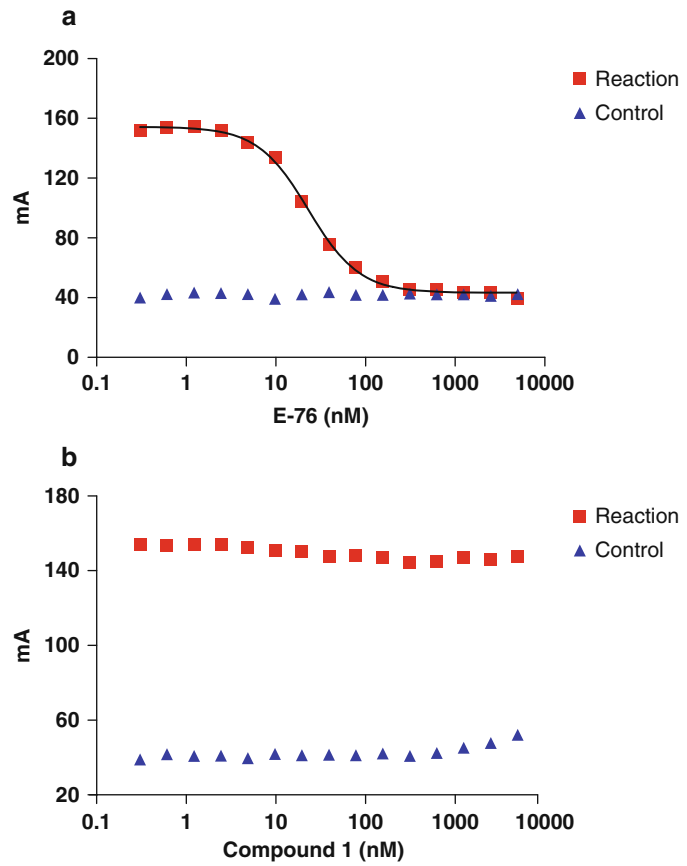

Fig. 6.5. The dose titration of E-76 and compound 1 in FVIIa FP competition assay. The assay contained 5 nM probe, 12.5 nM FVIIa, 37.5 nM sTF, and various concentrations of compound and was read for anisotropy on Analyst AD after 20 min incubation at RT. (**a**) E-76; (**b**) compound 1.

parallel to the competition reaction. The control reaction contains only the probe and the compound and is helpful in identifying compound interference issues.

A screen was performed at 300 μM using a set of compounds identified by virtual screening. A limited number of hits were identified and then followed up with dose titration in parallel with a control reaction as described above. Three hits were confirmed while the other hits were identified as false positives in the control titration due to compound interference including compound interactions with the probe, compound solubility/aggregation, and compound fluorescence. The three confirmed hits were followed up for further characterization.

2.1.5. A Quality Control Parameter for a Direct FP Competition Screen

A HTS campaign was run using one of the NR FP competition red assays from Invitrogen. The compound library was tested at 10 μM in singlet in a 20 μl assay in a 384-well black Nunc polypropylene

plate (cat# 267461). The screen was carried out on a Thermo-CRS platform and read on Envision plate readers (Perkin Elmer). A total of 24,256 hits were identified in the primary assay based on 3 × inter-quartile range standard deviation (IQR-SD) above the median. Next an interference screen (counter-screen) would be performed to determine if the hits are selective or just interfering in the assay. For direct FP competition assays there are no good options for a counter-screen. For this NR FP assay, the total fluorescence intensity (FLINT) was calculated from the S and P channel fluorescence and used for the calculation of a new quality control (QC) parameter, mP/total FLINT. We found that total fluorescence intensity of the bound probe is about 2 × that of the free probe in this assay, resulting in a relatively constant mP/total FLINT, regardless of the free and bound states of the probe. When mP/total FLINT is significantly below the median of the screen, the sample well likely contains a fluorescent compound. When mP/total FLINT is significantly above the median, the sample well either has a fluorescent quencher or has under-delivery of the probe. We did not identify many fluorescent compounds, likely due to the fact that the red probe was used in the assay. By applying a 3 × IQR-SD cutoff above the median for mP/total FLINT, we were able to eliminate 22% of the primary hits due to fluorescence quenching or probe under-delivery. The remaining primary hits were tested in a confirmation assay in triplicate and a large majority of hits (74%) were confirmed as active. Total fluorescence intensity has also been reported as a QC parameter for FP screens *(19)*.

2.2. FP as a Detection Method in Enzymatic Assays

Another application of FP assays in HTS is its use as a detection method in enzymatic assays. The detection methods can be divided into two types: (1) direct measurement of the product formation and (2) measurement of the product via FP competition. The pros and cons of these two types are discussed. Since these assays still measure FP, the standard practices for selection of the probe concentration, determination of K_d between the probe and the detection macromolecule, selection of the detection macromolecule concentration, optimization of reader protocols, optimization of plate, buffer, and other assay parameters as described earlier for direct FP competition assays (**Section 2.1**) still apply.

2.2.1. FP Method That Directly Measures the Product

The IMAP kinase and PDE assays (Molecular Devices) are examples of FP assays that directly measure the product of an enzyme reaction. In an IMAP kinase assay, the fluorescently labeled phosphopeptide product is detected by IMAP beads (IMAP beads serve as the macromolecules). The advantages of assays such as IMAP include the following: (1) measures the product directly and the

signal increases with the product; (2) compound potency (percent inhibition and IC50) does not change significantly with the assay conversion rate when the conversion rate is not greater than 50% *(20)*; and (3) an interference assay is available to remove false-positive hits due to interference with the detection system. The latter is easily accomplished by using the detection reagents plus the substrate and the product mixed at a ratio that mimics the amount of product formed during the assay. The disadvantages of IMAP assay include the following: (1) the use of labeled substrate instead of the "native" substrate and (2) the assay requires relatively higher conversion (20–50%) of substrate than conventional functional assays using more traditional detection schemes to achieve a reasonable assay mP window.

2.2.2. FP Method That Measures the Product via Competition

Transcreener PDE assay (Bellbrooke), PolarScreen kinase FP assay (Invitrogen), and PI3K FP assay (Echelon Biosciences) are examples of assays that measure the product via competition. In a Transcreener PDE assay, the product (GMP or AMP) is detected by competition with the fluorescently labeled probe for binding to the antibody. The advantages of competition detection assays such as Transcreener include the following: (1) uses the nonlabeled substrate and (2) interference assay is available to remove false-positive hits due to interference with the detection system (using the detection reagents plus the substrate and the product mixed at the assay conversion ratio). The disadvantages of competition detection assays include the following: (1) measures the product through competition and the signal decreases with increasing product; (2) it is difficult to accurately determine the Km for the substrate because the product standard curve is nonlinear due to nonlinear competition detection of the product; (3) the assay window is typically selected between the EC50 and the EC90 of the enzyme dose titration curve; however, compound potency (percent inhibition and IC50) can change significantly with the assay conversion rate. These disadvantages are universal to all competition detection assays and by no means unique to FP competition detection assays. For example, in a simple rapidly reversible competition model, the observed compound potency (IC50) determined from the competition assay deviates from the true IC50 (50% inhibition of the enzyme activity) (unpublished results, Huang, X., 2007). The observed compound IC50 is $2\times$ the true IC50 when the assay is conducted at EC50. However, the observed IC50 is $10\times$ the true IC50 if the assay is performed at EC90. The actual assay is a compromise of an acceptable assay signal window and workable assay sensitivity, knowing the caveat that a larger assay signal window comes at the price of possibly not identifying weakly active compounds.

2.3. Determination of Binding Mechanism from FP Competition-Binding Assay

The compound-binding mechanism can be derived from a compound dose titration in a FP competition-binding assay *(18)*. **Figure 6.6** shows three of the most common mechanisms for compound binding: competitive, uncompetitive, and noncompetitive binding. The α value in **Fig. 6.6** describes cooperativity between the probe binding and the inhibitor binding in a noncompetitive mechanism. A plot of the fraction bound versus compound concentration in **Fig. 6.7** shows the diagnostic displacement curves of compounds that bind competitively, uncompetitively, or noncompetitively to the macromolecule target *(18)*. A competitive inhibitor fully displaces the probe, resulting in a decrease in the

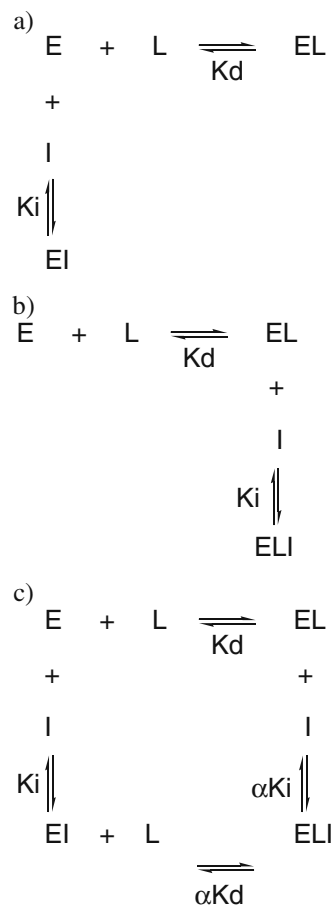

Fig. 6.6. Three common mechanisms for compound binding: (**a**) competitive mechanism; (**b**) uncompetitive mechanism; and (**c**) noncompetitive mechanism, where the α value describes cooperativity between the probe binding and the inhibitor binding. In a noncompetitive mechanism, $\alpha = 1$ represents no cooperativity between the probe binding and the inhibitor binding, $\alpha > 1$ represents negative cooperativity (the inhibitor binds weaker to the receptor–probe complex than to the free receptor), while $\alpha < 1$ represents positive cooperativity (the inhibitor binds stronger to the complex than to the free receptor). R is the receptor, L is the probe, and I is the inhibitor.

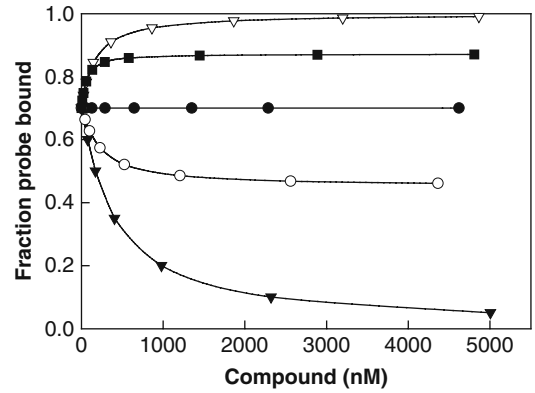

Fig. 6.7. Theoretical plots of competitive (▼), uncompetitive (▽), and noncompetitive ($\alpha = 1$ (▼), 3 (○), and 0.33 (■)) inhibitors. The receptor and probe concentrations are 53.7 and 10 nM, respectively. The K_d for the probe is 20 nM. The K_i for compounds in cases of competitive and uncompetitive mechanisms is 100 nM, while the K_i values in cases of noncompetitive mechanism are 100 and 100α nM.

fraction of ligand bound with increasing inhibitor concentration. In contrast, an uncompetitive compound increases the fraction of bound ligand, resulting in a higher polarization value. The displacement of probe by a noncompetitive compound is more complex and is dependent upon the degree of cooperatively. When the compound reduces ($\alpha > 1$), increases ($\alpha < 1$), or has no effect on probe affinity ($\alpha = 1$), the resulting fraction bound will decrease, increase, or not change, respectively (**Fig. 6.7**).

2.4. Limitations of Steady-State FP Measurements

As with any technique, fluorescence polarization has its share of limitations. General FP complications include interactions between the fluorescent probe and the compound, compound aggregation, scattered light, sample turbidity, plate or buffer polarization, compound fluorescence, fluorescence quenching due to various factors, and instrument detector saturation. Probe–target (or compound–target) interactions may also not fully mimic the native interactions, in cases including labeled probes having altered affinity/binding mode relative to unlabeled counterparts; using mutant enzymes in place of active enzymes; multiple enzyme conformations; and enzymes with multiple substrates. In addition, nonideal FP data can result from steady-state FP measurements of systems that possess multiple fluorescence lifetimes and/or multiple rotational correlation times and thus complex exponential decays, which may be accurately determined only by time-resolved FP measurements. Finally, because the FP assay signal window does not change significantly with the G factor and the starting polarization value is in practice set arbitrarily, the standard S/B parameter no longer indicates the robustness of the assay. For FP assays, the meaningful statistical parameters are assay signal window (mP) and Z′.

2.5. Summary

Fluorescence polarization is a powerful technique for the study of biomolecular interactions in solution and has been widely used in biochemical high-throughput screens. This chapter reviewed three representative steady-state FP applications and good practices in the development and execution of FP-based HTS assays. After hits are obtained from FP-based HTS assays, a good practice is to always confirm the hits in a secondary assay. For direct FP competition-binding assays, an orthogonal functional assay may be used. For FP detection assays, a second functional assay that employs a different detection method is recommended.

3. Notes

1. Fluorescence polarization measurements on commercial fluorescence plate readers follow one-photon excitation, which has a maximum anisotropy of 0.4. Anisotropy values higher than 0.4 indicate misalignment in the instrument or the presence of scattered light. Excitation with two photons or multiple photons by picosecond or femtosecond laser sources uses different photoselection processes, which can lead to a maximum anisotropy greater than 0.4 *(21)*.
2. The common practice is to set the instrument to 27 mP for 1 nM fluorescein and to subtract the background fluorescence (buffer only) from the I_S and I_P values as the background fluorescence is often polarized. We recommend adjusting the G factor of the instrument such that the probe mP is between 50 and 100 mP instead. Empirical results have shown that a FP assay signal window (probe bound minus probe free) does not vary significantly with the G factor. A higher initial mP for the probe can avoid situations in screens where polarization values are close to zero or turn negative. When the assay fluorescence intensity S/B is low (<20), background fluorescence due to buffer should be subtracted from the I_S and I_P values during calibration and buffer-only controls added to the assay plate. There are some commercial FP assays that have a FLINT S/B as low as 5. These assays are much more prone to larger CVs when implemented in screens without designated buffer-only wells. If the probe concentration yields a fluorescence intensity S/B > 50, background effects are minimized and background subtraction is not required.

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