TOPICAL REVIEW

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To cite this article: Matthew D Hall et al 2016 Methods Appl. Fluoresc. 4 022001

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**TOPICAL REVIEW**

Fluorescence polarization assays in high-throughput screening and drug discovery: a review

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Keywords: fluorescence polarization, anisotropy, high-throughput screening, assay development, enzyme, protein–protein interaction, drug discovery

Abstract

The sensitivity of fluorescence polarization (FP) and fluorescence anisotropy (FA) to molecular weight changes has enabled the interrogation of diverse biological mechanisms, ranging from molecular interactions to enzymatic activity. Assays based on FP/FA technology have been widely utilized in high-throughput screening (HTS) and drug discovery due to the homogenous format, robust performance and relative insensitivity to some types of interferences, such as inner filter effects. Advancements in assay design, fluorescent probes, and technology have enabled the application of FP assays to increasingly complex biological processes. Herein we discuss different types of FP/FA assays developed for HTS, with examples to emphasize the diversity of applicable targets. Furthermore, trends in target and fluorophore selection, as well as assay type and format, are examined using annotated HTS assays within the PubChem database. Finally, practical considerations for the successful development and implementation of FP/FA assays for HTS are provided based on experience at our center and examples from the literature, including strategies for flagging interference compounds among a list of hits.

1. Introduction

The screening of compound libraries to identify small molecule modulators of a biological target is an important and well-established paradigm for the discovery of novel therapeutics and molecular probes [1–3]. While a number of factors influence the selection of a primary assay technology to identify initial hits from compound libraries, orthogonal assay technologies are implemented as counter-screens to support target engagement and reveal false positive hits resulting from interference with the primary assay technology rather than modulation of the biological target [4]. Therefore, the drug discovery community relies on a variety of robust assay technologies that are both applicable to different types of biological processes and suitable for high-throughput screening (HTS). One such technology, fluorescence polarization (FP) is a powerful approach by which alterations in the apparent molecular weight of a fluorescent probe (or tracer) in solution are indicated by changes in the polarization of the sample’s emitted light [5]. The ability of FP assays to report on changes in molecular weight has been exploited to interrogate a variety of biological processes involving both molecular interactions (protein–protein, protein–peptide, protein–nucleic acid, protein–small molecule) and enzymatic activity (including substrate depletion and product formation) [6]. While FP measurements are often made from samples in a quartz cuvette, in 1996 Jolley was among the first to publish the implementation of FP in a microplate format in order to measure inhibitor activity against the proteolytic cleavage of BODIPY-labeled α-caseins [7]. Since FP was first applied to screening in the 1990s, the methodology has advanced substantially and been applied to increasingly complex biological systems [8]. Advantages of the FP assay approach include the ability to study molecular processes in solution, a homogenous (all-in-one) format, comparatively low cost, the ability to read multiple times (timecourse) and a relative insensitivity to some types of assay interferences, such as inner filter effects, that hinder other light-based assay technologies. Several recent articles provide detailed protocols for the development of HTS FP assays based on the 384-well format [9, 10]. This review is intended to complement these articles by focusing on trends in...
FP assay development and practical considerations for the optimization and miniaturization of assays to the ultra-high-throughput 1536-well format.

1.1. Principles of fluorescence polarization and anisotropy

In 1926, Francis Perrin described the theoretical basis of fluorescence polarization based on the observation that the emission from a small fluorescent molecule excited by plane-polarized light is largely depolarized due to rotational diffusion during the lifetime of the fluorescence [11]. Perrin derived equation (1), where FP is the observed polarization, R is the universal gas constant, T is the absolute temperature, η is the solution viscosity, FP₀ is the intrinsic polarization (polarization value in the absence of molecular rotation or FP when T/η → 0), V is the molar volume and τ is the lifetime of the excited state of the fluorescence. Equation (1) clearly illustrates how the observed fluorescence polarization depends on the molecular volume of the fluorescent species when the temperature and solution conditions are constant. Therefore, fluorescence polarization can be used to follow biological processes that involve changes in molecular weight, such as a binding event or the enzymatic cleavage of a substrate (figure 1). The rotational correlation time (θ) for a molecule, shown by (2), contributes to (1) in that FP increases as θ increases. From (1), it is also clear that FP decreases as τ increases.

\[
\frac{1}{FP} - \frac{1}{3} = \left( \frac{1}{FP_0} - \frac{1}{3} \right) \left( 1 + \frac{RT}{\eta V} \tau \right)
\]  

\[\theta = \frac{\eta V}{RT}\]  

In the 1950s, Weber extended Perrin’s theoretical work to ellipsoids of revolution, developed the first instrumentation to measure fluorescence polarization, and applied it to the study of proteins [13–15]. To measure fluorescence polarization in the laboratory, a fluorescent sample is excited by polarized light and emission intensities are collected from channels that are parallel (Iǁ) and perpendicular (I⊥) to the electric vector of the excitation light (figure 2). These measurements can be used to calculate either fluorescence polarization (FP) or fluorescence anisotropy (FA, explained below), which are both widely used. Equation (3) shows the calculation of FP, which is quantified as the difference between the parallel (Iǁ) and perpendicular (I⊥) emission intensities normalized by the total fluorescence intensity of the emission beam.

\[FP = \frac{Iǁ - I⊥}{Iǁ + I⊥}\]  

Fluorescence anisotropy (4) is defined as the difference between the parallel (Iǁ) and perpendicular (I⊥) emission intensities normalized by the total fluorescence intensity from the sample.

\[FA = \frac{Iǁ - I⊥}{Iǁ + 2I⊥}\]  

Anisotropy does not provide any additional information over polarization and both functions are commonly utilized. Anisotropy is sometimes preferred because deconvolution of anisotropy into its component values appears simpler than for polarization, although the differences are minor [16, 17]. Additionally, the total fluorescence intensity calculated as the denominator of (4) is useful for flagging interference compounds as discussed in more detail below. In any case, equations (5) and (6) can be used to interconvert between fluorescence polarization and anisotropy values and the remainder of this article will focus on FP, which is more frequently referred to in the PubChem BioAssay database.

\[FA = \frac{2FP}{(3 - FP)}\]  

\[FP = \frac{3FA}{(2 + FA)}\]  

Due to the geometry of microplates, plate reading instruments are unable to measure light transmitted at a 90° angle from the excitation axis as would be performed with a quartz cuvette (figure 2). Instead, the excitation light reaches the sample after passing through a hole in a mirror that directs the emission light towards the detector optics [18]. The quantitative value of FP is a dimensionless number because it is a ratio of light intensities, but it is typically expressed in millipolarization units (mP) as shown from (7). A direct comparison of FP data collected from different instruments requires a calibration step because instruments vary in their sensitivity for measuring emission intensities from the Iǁ and I⊥ channels. This is accomplished by a grating factor or ‘G-factor’ to correct for any bias towards or against the I⊥ channel (7). A G-factor of 1 indicates no bias, while a G-factor >1 corrects a bias against I∥ and a G-factor <1 corrects a bias towards I⊥. Typically the calibration is performed by measuring a 1 nM fluorescein solution at room temperature and adjusting the G-factor to achieve a value of 27 mP [19].

\[FP (mP) = \frac{(Iǁ - I⊥ × G)}{(Iǁ + I⊥ × G)} × 1000\]  

Although the absolute value of FP can vary significantly with the G-factor, the dynamic range of an assay (free probe compared with complexed probe) remains fairly constant because it depends on changes in FP values rather than absolute measurements. After making a measurement, the background fluorescence from the buffer should be subtracted from the Iǁ and I⊥ channels. Typically the suitability of an assay for HTS is evaluated based on the Z’ factor, which reflects the separation in mean values for the high and low controls while taking into consideration the variability within each group [20]. Equation (8) shows how to calculate the Z’ factor, where the mean values and standard deviations for the high and low controls are indicated by μ+, σ+ and μ−, σ−, respectively.
A Z′ factor value below zero indicates a poor quality assay with no separation between the high and low controls. Assays with a Z′ factor of zero can work as a ‘yes/no’ approach, but there is no separation band between the high and low controls, which results in the identification of both false positive and false negative hits. A Z′ factor value between zero and 0.5 indicate a small separation band, whereas a Z′ factor value between 0.5 and 1 indicate an excellent quality assay.
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with a large separation band between high and low controls. Preferably, optimized FP assays have $Z'$ values above 0.5, which is well-suited to HTS.

2. Applications of fluorescence polarization in drug discovery

The principles of FP have been applied extensively in assays to enable high-throughput screening of small molecule libraries for the purposes of probe development and drug discovery. A search of the PubChem BioAssay database for ‘fluorescence polarization’ and ‘fluorescence anisotropy’ on October 4, 2015, identified 1000 unique FP and FA assays. Among the list, 834 assays were annotated according to how they were implemented in an HTS campaign. While 15% of these HTS assays were utilized for primary screens, 85% were implemented as counter or orthogonal screens to identify false positive hits and confirm activity against the target. Figure 3 shows that FP assays have been developed to interrogate an impressive diversity of biological target classes, including receptors, ion channels, epigenetic regulators, transcription factors, kinases, proteases and isomerases. This highlights the utility of FP assays to report on a range of critical biological activities for HTS applications. In this section, we briefly discuss different types of FP assays implemented in HTS from the recent literature to emphasize the multitude of approaches to assay development.

One major application of FP assays is the interrogation of biologically relevant molecular interactions, either due to direct binding of a fluorescently labeled molecule or through competition with an unlabeled species. Such approaches have enabled assays that report on receptor-ligand, protein–peptide, protein–protein and protein–nucleic acid interactions. For example, Wang and colleagues recently reported an HTS assay with a FP readout to identify inhibitors of the interaction between human antigen R (HuR) and RNA [21]. The HuR protein stabilizes mRNA molecules of proto-oncogenes, transcription factors, cytokines and growth factors and increased HuR expression is linked to carcinogenesis and correlates with poor outcome. Reducing the expression of HuR by knockdown increased the sensitivity of cells to chemotherapeutics and promoted apoptosis. Therefore, HuR has been identified as a potential target for cancer therapeutics. In order to identify molecules that destabilize the HuR/RNA interaction, a competition assay was developed using 5′-FAM (fluorescein) labeled 11-mer polyribonucleotide and full-length HuR protein at concentrations to yield FP values at ~80% of the maximum (100% bound RNA). Displacement of the tracer-labeled RNA by unlabeled RNA or inhibitor compounds resulted in reduced FP and the optimized assay had a dynamic range of about 100 mP and an average $Z'$ factor value of about 0.8 among 5 plates. After screening the NCI diversity set V library of 1,597 compounds (https://wiki.nci.nih.gov/display/NCIDTPdata/Compound+Sets) with the FP primary assay, the authors identified compounds that disrupted the HuR/RNA interaction as well as HuR oligomerization.

Similarly, the development of FP assays based on peptide–protein binding has been a powerful approach in screening for inhibitors of molecular interactions. A recent example of this is a primary screen to identify chemical antagonists of the anti-apoptotic Bcl-2 family protein member, Bfl-1 [22]. Bfl-1 is overexpressed in some malignant cells and contributes to chemoresistance and tumor cell survival. Bfl-1 and other anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-XL, Bcl-W, Bcl-B and Mcl-1) interact with BH3-only pro-apoptotic family members (including Bid and Bim) to regulate the pro-apoptotic multidomain Bcl-2 family proteins Bax and Bak. A competitive FP assay was developed using fluorescein isothiocyanate (FITC)-conjugated Bid BH3 peptide and a glutathione S-transferase (GST)-tagged Bfl-1 protein lacking the C-terminal transmembrane domain. The complex of GST-Bfl-1 with the FITC-Bid BH3 peptide produced...
AMP2/GMP2 fluorescence polarization reagent from other enzymatic reactions, such as the Transcreener available to measure the products of kinases as well in a lower FP value.

substrate, which displaces the labeled peptide, resulting in a high FP value that was decreased following displacement of the AMP-tracer from the antibody, resulting in a high polarization signal. Screening 2880 compounds with the assay enabled the identification of a new RNase P inhibitor. In another recent example, a FP assay was utilized to discover inhibitors for the cleavage of the human immunodeficiency virus (HIV) Gag protein into its two structural proteins, matrix (MA) and capsid (CA) [33]. Instead of targeting the protease, the authors wanted to discover molecules that target the Gag protein substrate and prevent its proteolysis. The assay substrate was a 56 kDa MA/CAΔ fusion protein with GST at the N-terminus and a tetracysteine motif (CCPGCC) within CA to facilitate fluorescent labeling by the fluorescein arsenical hairpin (FlAsH) reagent [34]. The substantial depolarized emission of the 15 kDa proteolytic product, relative to the full-length fusion protein, resulted in a large dynamic range and Z’ values above 0.85 during assay validation. A library of 24 000 compounds was screened with the validated protease assay. The low hit rate of 0.11% with a species of salivary trypanosome that causes human African trypanosomiasis [30]. Similarly, Peterson and colleagues recently reported using the Transcreener reagent in a primary assay developed to identify inhibitors of the purified Escherichia coli RecA protein, which is a DNA-dependent ATPase essential for DNA repair and important for bacterial survival and resistance to antibiotics [31]. Previously reported assay approaches required high concentrations of protein (≥5 μM) and ATP (≥0.75 mM), which drove RecA almost exclusively into an active DNA-bound conformation and favored the identification of inhibitors selective for the active conformation. The authors reasoned that a sensitive assay capable of detecting low ATP turnover with reduced enzyme and substrate concentrations might allow the identification of inhibitors selective for either the active or inactive conformations of RecA by eliminating a bias towards the active conformation. Additionally, the reduced enzyme and substrate concentrations would lower the stoichiometric limit on measured IC50 values and allow the identification of ATP competitive inhibitors. The optimized assay was utilized to screen a library of 113 477 compounds with an average Z’ value of 0.92. Of the 177 confirmed hits, 35 small molecules were active in bioassays with live bacteria.

When the result of an enzymatic reaction is the degradation of a large molecule into smaller fragments, it can be possible to develop a FP assay to follow the reaction based on the difference in polarization of the larger labeled substrate compared with the cleaved labeled product. This approach has been used to target a variety of degradative enzymes, including nucleases and proteases. Recently, this approach was applied to develop a FP assay to identify inhibitors of Ribonuclease P (RNase P), an essential endonuclease, purified from Bacillus subtilis [32]. The assay measures RNase P activity against a 5’ fluorescein-labeled pre-tRNA Ap substrate that has a high polarized signal. Upon cleavage, the fluorescein-5nt-leader product is released, resulting in a depolarized emission. Screening 2880 compounds with the assay enabled the identification of a new RNase P inhibitor. In another recent example, a FP assay was utilized to discover inhibitors for the cleavage of the human immunodeficiency virus (HIV) Gag protein into its two structural proteins, matrix (MA) and capsid (CA) [33]. Instead of targeting the protease, the authors wanted to discover molecules that target the Gag protein substrate and prevent its proteolysis. The assay substrate was a 56 kDa MA/CAΔ fusion protein with GST at the N-terminus and a tetracysteine motif (CCPGCC) within CA to facilitate fluorescent labeling by the fluorescein arsenical hairpin (FlAsH) reagent [34]. The substantial depolarized emission of the 15 kDa proteolytic product, relative to the full-length fusion protein, resulted in a large dynamic range and Z’ values above 0.85 during assay validation. A library of 24 000 compounds was screened with the validated protease assay. The low hit rate of 0.11% with...
this assay resulted in the identification of few false positive hits, which make this assay well suited for screening larger compound libraries.

Fluorescence polarization–activity-based protein profiling (fluopol-ABPP) is a substrate-free method that can be applied to uncharacterized enzymes [35]. Covalent linkage of a reactive fluorescent probe to the active site of an enzyme results in a higher FP measurement. Library compounds that bind the active site and block labeling by the activity-based probe are indicated by lower FP values. This methodology has enabled the identification of inhibitors for a number of enzymes from HTS of large chemical libraries [36–41]. Most recently, Chang and colleagues utilized fluopol-ABPP to identify inhibitors of the platelet-activating factor acetylhydrolases (PAFAHs) 1b2 and 1b3, which are catalytic subunits that associate in a heterotrimeric complex with the noncatalytic protein, 1b1 [42]. Selective inhibitors of PAFAH1b2/3 could help elucidate the catalytic and non–catalytic functions of the PAFAH1b complex. Both PAFAH1b2 and 1b3 are serine hydrolases and the serine nucleophile of the serine-histidine-aspartic acid triad was targeted for covalent modification by a labeled fluorophosphonate inhibitor. Selective and reversible inhibitors were identified from a library of >300 000 compounds and one of the inhibitors reduce the survival of human and mouse cancer cell lines.

3. Case studies of high-throughput fluorescence polarization assays

Given the frequent use of FP assays at the National Center for Advancing Translational Sciences (NCATS), we have selected several recent examples to use as case studies. These examples are not comprehensive, but allow for a summary of the basis or scientific rationale of the work, the specific assay that was developed, and the results and limitations of the study.

3.1. Understanding small molecule protein binding interactions

A critical component for lead development in any drug discovery program is the assessment of candidates using ADMET assays. One crucial parameter for drug or image probe activity is the free fraction of a chemical probe in blood plasma (f<sub>p</sub>), which is primarily governed by blood plasma protein binding [43]. The two most important proteins involved in plasma drug binding are human serum albumin (HSA) and α-acid glycoprotein (AGP). Well-established methods for assessing plasma protein binding are relatively laborious and rely on equilibrium binding of the compound in plasma, followed by separation of the protein by techniques such as ultrafiltration. Furthermore, measurement of compounds relies on chromatographic separation or utilizing radiolabeled compounds, rendering the process low-throughput [44].

In an effort to allow assessment of a larger set of compounds from a medicinal chemistry program or to profile drug libraries, Mathias et al developed a FP assay for protein binding, which was subsequently miniaturized to a 1536-well format [45, 46]. Given that FP requires the assessment of a specific molecular interaction, a serum-based binding assay was not feasible, so the investigators developed two separate assays for binding to HSA and AGP. Albumin contains multiple defined drug binding sites, with the two most common referred to as site I and site II. For each protein, a known small–molecule binder was selected as a tracer that is inherently fluorescent; for HSA it was dansyl sarcosine (a site II binder), and for AGP it was dipryramidole.

The basis of the assay is that FP will increase when the fluorescent small molecule probe is non-covalently bound to the protein. Equilibrium displacement of the protein-bound probe by a test compound will result in reduced FP, due to the faster rotation of the unbound probe. Optimization of the two assays were virtually identical, so only the HSA assay optimization is described here. By titrating dansyl sarcosine against HSA and measuring anisotropy, a K<sub>d</sub> value of 0.26 μM was determined. A competition assay with dansyl sarcosine (500 nM, approximate K<sub>d</sub>) against six non–fluorescent ligands known to bind HSA site II were examined (HSA concentration was 5 μM), as well as a site I binder as a negative control, resulting in displacement curves with EC<sub>50</sub> values ranging from 50–10 000 μM. Based on a 130-fold difference in displacement for the site II competitor, naproxen, compared with the negative control, phenylbutazone, it was concluded that dansyl sarcosine is a specific probe for HSA site II binding. A small-scale screen of 88 compounds for HSA binders, with naproxen as the positive control, was performed in the 96-well format, resulting in a Z′ value of 0.83.

Subsequent implementation of the HSA and AGP binding assays in 1536-well format followed. For assay miniaturization, a matrix titration of dansyl sarcosine and HSA was conducted to optimize the dynamic range. Concentrations of 3 μM dansyl sarcosine and 10 μM HSA were selected, which resulted in a Z′ value of 0.75 in the 1536-well format. Similarly, an optimal combination of dipyridamole (1 μM) and AGP (4 μM) was determined (figure 4(a)) with an identical Z′ value. A 10-compound validation set was tested in 1536-well format for each protein, resulting in data comparable to the 96-well format. The Library of Pharmacologically Active Compounds (LOPAC, 1280 compounds) was screened against both assays, with a concentration window of 457 nM–144 μM (AGP) and 3.1–154 μM (HSA), and known binders of each protein were identified from the screen.

These assays provided the first screening strategy for the major drug-binding proteins in human blood plasma. There are a number of considerations for this assay strategy (aside from the standard cautions relating to interference from fluorophores in the library). Given the utilization of mouse models in pharmacology, insight into species differences between mouse
and human HSA and AGP would be interesting. Future studies might also address whether positive hits from the FP assay could be recapitulated using human serum diluted appropriately. Finally, a limitation of the HSA probe is that the protein has multiple binding sites, whereas the probe, by definition, binds to only one. As such, a test compound that is not active in the HSA assay does not rule out binding to another site. The authors followed this work by developing a non-FP-based displacement assay for HSA that used the fluorescent small molecule, Red Mega 500, whose fluorescence is quenched upon binding to HSA [47].

3.2. Inhibition of the interaction between the vitamin D receptor (VDR) and steroid receptor coactivator 2

The vitamin D receptor (VDR) is a ligand-activated transcription factor (nuclear receptor) that plays a role in regulating the endocrine system. This includes the transcriptional regulation of genes for the production of parathyroid hormone (PTH) and the regulation of blood Ca\(^{2+}\) levels. The VDR-mediated gene regulation, activated by its ligand, 1,25-dihydroxyvitamin D\(_3\) (\(1,25-(\text{OH})_2\text{D}_3\)), is governed by the recruitment of coactivators and VDR has been identified as a potential target for the treatment of a range of disorders, including metabolic disorders, cancer, autoimmune disease and cardiovascular disease. Interactions between VDR and coregulators are critical for VDR-mediated transcriptional activation [48].

Nandhikonda et al designed a FP assay to screen for inhibitors of the interaction between VDR and one such coregulator, steroid receptor coactivator 2 (SRC2). The authors produced an Alexa Fluor 647-labeled SRC2 peptide called SRC2-3 (CLQEEKRLHKLQNGNSPA, \(\lambda_{\text{EX}} = 630\) nm/\(\lambda_{\text{EM}} 685\) nm) previously characterized as having a strong affinity for the VDR (\(K_d = 174\) nM) [49, 50]. A far-red fluorophore was selected to avoid fluorescence interference. While the assay optimization is not described in detail, the original HTS was carried out using 384-well plates and a purified fusion protein of maltose binding protein with the VDR ligand binding domain (MBP-VDR-LBDmt). The MBP component aided in solubility of the VDR domain and did not interact with the SRC2 peptide [50]. Additionally, the synthetic VDR agonist, LG190178, was included in the assay because VDR agonist binding is necessary to allow coregulator (SRC2) binding. Therefore, assay reagents included MBP-VDR-LBDmt protein (1 \(\mu\)M), LG190178 (5 \(\mu\)M), and Alexa Fluor 647-labeled SRC2-3 (7.5 nM) (figure 4(b)). Approximately 275,000 small molecules were tested at a single concentration of 30 \(\mu\)M. After 3 h of incubation, FP was measured with the test
compounds and any binding was expected to result in a loss of polarization due to the displacement of the fluorescent peptide. A set of 589 compounds exhibiting more than 40% inhibition were re-tested and resulted in a final set of 29 high-quality active compounds. Of the chemotypes identified from the HTS, 3-indolylmethanamines were selected for follow-up medicinal chemistry efforts. The FP assay was also used for assessing compounds synthesized as part of a medicinal chemistry campaign, and while improved analogs were generated, the compound class was found to act via irreversible inhibition of the VDR and to be selective among six other NR-coactivator interactions tested [49].

A follow-up HTS was performed in 1536-well format with 390 000 compounds screened at multiple dose-points [51]. Hits from the screen were validated with two alternative FP assays using SRC2-3 labeled with Texas Red or fluorescein. Given that the original screen focused on a hit that covalently/irreversibly reacted with the VDR, a counter-screen was employed to triage reactive hits. This was achieved by conducting the FP assay in the presence of high concentrations of β-mercaptoethanol (BME) [52]. The expectation was that thiol-reactive compounds will preferentially react with the overwhelming concentration of BME, resulting in loss of target inhibition. A large number of hits were prevented from inhibiting the VDR–SRC2-3 interaction under these conditions. The remaining hits included GW0742, a compound produced by GlaxoSmithKline under these conditions. The authors selected assay concentrations of 2.5 μM S100B with 50 nM TAMRA-TRTK, for a fractional occupancy of 0.67 (figure 4(c)). It is interesting to note that the assay buffer incorporated 5% DMSO and 0.1% Triton X-100 to favor compound solubility at the high concentrations expected to disrupt the S100B-TAMRA-TRTK binding interface.

The FP assay was screened against the Spectrum Collection library at four concentrations (62.5, 31.3, 15.6, and 7.8 μM), with 49 possible S100B inhibitors emerging after triaging compounds suspected of contributing fluorescence interference. These compounds were evaluated by secondary screening using NMR to monitor protein 1H and 15N chemical shifts in the presence of putative inhibitors and Kd values for inhibitor binding were determined using the FP assay. The authors found that some hits initially eliminated due to expected fluorescence interference did in fact bind with S100B in the NMR assay. Crystal structures of S100B were then determined and the screen hits chlorpromazine, thimerosal, and sanguinarine were co-crystalized and found to bind in the p53-binding pocket. The latter two compounds are known reactive molecules and were shown to be bound with cysteine residues in the protein. The incorporation of structural insight into the FP assay workflow clearly empowered hit validation and mechanistic understanding.

A follow-up screen was conducted, continuing the NMR-guided interrogation into target binding, with a focus on identifying hits that bind specifically to a cysteine residue in site 2 of S100B, which could be used as a basis for a medicinal chemistry campaign, generating inhibitors that occupy sites 1, 2, and 3 simultaneously [61]. The LOPAC library of 1280 compounds was screened in dose-response, identifying two further covalent inhibitors in addition to those identified from
the first study. All five compounds were used for structure-function characterization by x-ray crystallography and medicinal chemistry. It is important to note that the hits in this assay are technically PAINS (Pan Assay Interference Compounds) by vice of their inherent reactivity [62]. However, in the context of desired activity, these reactive compounds form the basis for future medicinal chemistry development of inhibitors for the S100B-p53 interaction.

4. Developing fluorescence polarization assays

Due to the diversity of possible approaches that could be applied to the development of FP assays suitable for the study of enzymes and molecular interactions, a comprehensive discussion of FP assay development for HTS is beyond the scope of this article. Recently, detailed methods and protocols for FP assay development in a 384-well format have been reported [9, 10]. The remainder of this review will examine some of the common practices in FP assay development for HTS and offer practical advice for the development of assays based on experience at our center, primarily involving the ultra-high-throughput 1536-well format.

The process of developing a FP assay suitable for HTS can require a number of optimizations in the labeling strategy and assay conditions as described below in more detail. Therefore, it is worth considering early on whether commercially available products might be applied to the target of interest. Table 1 lists a number of currently available products for fluorescence polarization assays. While some of the products are focused on particular targets, others can be applied more generally. For example, the Transcreener assay can be applied to study enzymatic activities that are coupled to nucleotide hydrolysis. Due to the coupling of an enzymatic assay to a common reaction product, such as ADP, commercially available reagents might be more easily applied to enzymatic targets. However, a FP assay involving molecular interactions (e.g. protein-small molecule, protein-protein, protein-peptide or protein-nucleic acid) often requires the complete development, validation and optimization of an assay. In this case, an important initial consideration is the affinity of the targeted interaction. Consider a reversible, bimolecular association between a probe-labeled ligand (L) and a protein (P) with a 1 : 1 stoichiometry as shown in (9).

\[
P + \frac{1}{K_d} \rightarrow \text{PL} \quad (9)
\]

According to the law of mass action, the equilibrium dissociation constant \(K_d\) is represented by (10).

\[
K_d = \frac{[P][L]}{[PL]} \quad (10)
\]

Typically for a competition assay, \(L\) (the smaller labeled molecule) is added at a concentration matching the \(K_d\) value (that is, the concentration at which \(L\) is 50% free (L) and 50% bound to protein (PL)), and P (the larger unlabeled molecule) is present at a concentration that allows 50–80% of the ligand to be bound [63]. The concentration of bound complex (PL) can be calculated from (11) if the \(K_{d, L}\), total ligand (LT) and total protein are known (Pr).

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
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<th>Vendor</th>
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<td>IMAP®</td>
<td>384-well</td>
<td>Molecular devices</td>
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<td>Kinase</td>
<td>PI3-Kinase fluorescence polarization activity assay</td>
<td>384-well</td>
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<td>Direct detection of nucleotide enzyme products</td>
<td>Transcreener HTS assays: ADP², AMP²/GMP², GDP, UDP</td>
<td>1536-well</td>
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<td>Predictor™ hERG fluorescence polarization assay kit</td>
<td>384-well</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>Phosphodiesterases (PDEs)</td>
<td>Phosphodiesterases (PDEs)</td>
<td>96-well</td>
<td>BPS Bioscience</td>
</tr>
<tr>
<td>Molecular Chaperones</td>
<td>HSP90 assay kit</td>
<td>96-well</td>
<td>BPS Bioscience</td>
</tr>
<tr>
<td>Oxidative Stress</td>
<td>Keap1:Nrf2 inhibitor screening assay kit</td>
<td>96-well</td>
<td>BPS Bioscience</td>
</tr>
</tbody>
</table>
Figure 5. Requirements for the protein (P) and ligand (L) concentrations in a FP assay depend on the binding affinity. The percent bound ligand was calculated for reversible 1:1 interactions using equation (11) and fixing the total ligand concentration at the $K_d$ value. The plot shows theoretical binding curves for interactions with $K_d$ values of 1 nM (red solid line), 10 nM (orange dashed line), 100 nM (green dotted line), 1 μM (blue dot-dash line) and 10 μM (violet dash-dot-dot line). Typical FP competition assays are optimized with the ligand concentration near the $K_d$ value and a protein concentration resulting in between 50–80% of the ligand bound (black dotted lines).

$$[PL] = \frac{(K_d + L + P_T) - \sqrt{(K_d + L + P_T)^2 - 4P_TL}}{2}$$

(11)

Figure 5 shows simulated binding curves for reversible heteromeric 1:1 interactions of different affinities (assuming a 100% binding competency for both components). In each case, the concentration of L is fixed at the $K_d$ value and the concentrations of P are varied. Strong and weak interactions present different challenges to assay development. While strong affinity interactions (e.g. $K_d = 1$ nM) require relatively low concentrations of P (~1.5–5 nM) and L, the experimental noise in the polarization measurements is increased due to the low fluorescence intensity signal. Also, the polarization signal from a low concentration of labeled L can be masked by some library compounds that are typically introduced at much higher concentrations (>1 μM). In this case, one might choose a red-shifted probe to reduce fluorescence interference (described in more detail below). On the other hand, weak affinity interactions require higher probe concentrations, which can reduce interferences by library compounds, but are associated with other challenges, including the potential for the probe to aggregate at higher concentrations. Additionally, weaker affinity interactions, for instance with a $K_d$ of 10 μM (figure 5, violet dash-dot-dot line), require higher concentrations of unlabeled protein (~15–50 μM) in order to yield an adequate fraction of bound probe, which might limit the feasibility of a screen. For example, with a 20 kDa protein in a FP assay optimized for 4 μl samples (1536-well plates) or 16 μl samples (384-well plates), an affinity of $K_d = 10$ μM would theoretically require 1.8–6 mg of protein per plate, whereas an affinity of $K_d = 1$ nM would require 200–600 ng of protein per plate. In this scenario, the stronger affinity interaction enables 10 000 plates to be screened with the same quantity of P required for 1 plate with the weak affinity interaction.

Miniaturization of an assay can substantially reduce the amount of materials required to screen library compounds. At our center, we routinely utilize a quantitative high-throughput screening (qHTS) approach with the 1536-well format, where compounds are tested over a broad concentration range from micromolar to low nanomolar [64]. In addition to reducing the quantity of each reagent required to perform a screen, this format also offers a number of other advantages for HTS. For instance, the efficacies and potencies of active compounds are available directly from the primary screen. Additionally, it is easier to identify single-point outliers among a titration series, which reduces the rate of both false negative and false positive hits. The higher plate density also enables the application of controls such as an intra-plate titration, in addition to the normalization controls. Finally, assay miniaturization reduces interferences caused by absorption, which will be discussed in more detail below [65]. A survey of 210 annotated HTS FP assays from the PubChem database indicates that 79% utilized the 1536-well format (figure 6).

4.1. Selection of an appropriate fluorescent probe

Developing a fluorophore-labeled ligand for a FP assay requires consideration of multiple factors as well as experimental validation. Numerous fluorophores have been utilized for FP assays and selecting a fluorophore is the first step in the process of assay development. Properties that should be considered include fluorescence lifetime, quantum yield, extinction coefficient, and stability. The preferred fluorescence lifetime ($\tau$) of a probe for any assay will depend on $\theta$ of the unbound ligand and the change in molecular weight after binding. Together, these properties determine the dynamic range of a FP assay. If $\tau$ is substantially shorter than $\theta$ of the unbound ligand, the emission is
largely polarized in the unbound state, which reduces
the dynamic range of the assay. Conversely, if \( \tau \) is
significantly longer than \( \theta \) of the molecular complex,
the emission will be largely depolarized in the bound
state, resulting in low FP values. The simulations in
figure 7 show how \( \tau \) and molecular weight contribute
to measured FP values. Free probes in solution can
have \( \theta \) values in the range of hundreds of picoseconds,
compared to tens of nanoseconds for larger molecular
complexes. As a rough approximation, \( \theta \) can be
estimated at 0.4 ns kDa\(^{-1}\) for spherical proteins in
typical buffers at room temperature [65, 67]. Small
ligands containing fluorophores that have \( \tau \) values
between 1–6 ns are often successfully paired with
round proteins with molecular weights less than
10 kDa; however, ligands with larger molecular weights
typically require fluorophores with longer lifetimes.
The dynamic range for FP assays intended to detect
molecular interactions between proteins might be
increased by expressing the unlabeled protein as a
chimera with another protein, such as GST, to increase
the change in molecular weight upon binding [23].

The quantum yield (\( \phi \)) of a fluorophore is cal-
culated as the ratio of the number of photons emitted to
the number of photons absorbed [5]. Higher quantum
yields are typically preferred for probes, particularly
in the case of strong affinity interactions that require
low probe concentrations. Depending on the rigidity
of the excited state geometry, the fluorescence prop-
erties of some probes can vary with the local environ-
ment. Probes that have rigid excited state geometries,
such as fluorescein, do not show significant changes
in emission between the free and ligand bound states;
however, probes with flexible excited state geometries
behave differently. Alterations in quantum yield upon
ligand binding to a target complicate the calculations
of fraction of ligand bound and can cause errors in the
determination of \( K_d \). Such changes can be corrected for

Figure 6. Distribution of FP plate formats among HTS assays. A pie graph shows the distribution of plate formats indicated by
210 PubChem assay IDs. The BioAssay Research Database (BARD) was queried to gather assay annotations for a collection of
fluorescence polarization assays [66]. The 1536-well plate format was the most common (166 or 79%), followed by 384-well
(42 or 20%) and 96-well plates (2 or 1%), respectively.

![Figure 6](image)

Figure 7. The theoretical relationship between molecular weight and fluorescence polarization for different fluorescence lifetime
(\( \tau \)) values. Simulations are shown for probes with \( \tau \) values of 1 ns (blue solid line), 4 ns (green dashed line), 6 ns (orange dotted line)
and 20 ns (black dot-dash line). The simulations assume a \( P_0 \) value of 0.5 and rigid attachment of probes to spherical molecules.

![Figure 7](image)
by taking the change in quantum yield into account by equation (12), where \( f_b \) is the fraction of bound ligand, \( F_P_b \) is the polarization of the bound ligand, \( F_{P_{obs}} \) is the observed polarization, \( F_P_f \) is the polarization of the ligand free in solution and \( g \) is the quantum yield enhancement factor [17].

\[
f_b = \frac{(3 - F_P_b)(F_{P_{obs}} - F_P_f)}{(3 - F_{P_{obs}})(F_P_b - F_P_f) + (g - 1)(3 - F_P_f)(F_P_b - F_{P_{obs}})}
\]  

Figure 8. Distribution of FP probes among annotated HTS assays. A pie graph shows the different fluorescent probes utilized among 213 unique PubChem assay ID numbers. The BioAssay Research Database (BARD) was queried to gather assay annotations for a collection of fluorescence polarization assays [66]. Among these annotated assays, fluorescein dyes are the most common fluorescent probe, followed by rhodamine dyes and Cy5. The graph is colored according to the emission maxima of the fluorophores. Fluorophore structures were generated using the program Chem & Bio Draw (CambridgeSoft).

Table 2. Properties of the FP probes from figure 8.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
<th>( \tau ) (ns)</th>
<th>( \varphi )</th>
<th>( \varepsilon ) (cm(^{-1}) M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>BODIPY FL</td>
<td>502(^a)</td>
<td>510(^a)</td>
<td>5.7(^a)</td>
<td>NA</td>
<td>82 000(^a)</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>494(^a)</td>
<td>519(^a)</td>
<td>3.8(^a)</td>
<td>0.93(^a)</td>
<td>77 000(^a)</td>
</tr>
<tr>
<td>Alexa 488</td>
<td>495(^a)</td>
<td>519(^a)</td>
<td>4.1(^a)</td>
<td>0.92(^a)</td>
<td>73 000(^a)</td>
</tr>
<tr>
<td>Acridine Orange</td>
<td>460(RNA)/500(DNA)(^a)</td>
<td>650(RNA)/526(DNA)(^a)</td>
<td>2.0(^b)</td>
<td>NA</td>
<td>53 000(^a)</td>
</tr>
<tr>
<td>Alexa 532</td>
<td>531(^a)</td>
<td>554(^a)</td>
<td>2.5(^a)</td>
<td>0.61(^a)</td>
<td>81 000(^a)</td>
</tr>
<tr>
<td>Cy3B</td>
<td>558(^b)</td>
<td>572(^b)</td>
<td>2.8(^b)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5-TAMRA</td>
<td>542(^a)</td>
<td>568(^a)</td>
<td>NA</td>
<td>NA</td>
<td>91 000(^a)</td>
</tr>
<tr>
<td>Texas Red</td>
<td>595(^a)</td>
<td>615(^a)</td>
<td>4.2(^b)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Alexa 594</td>
<td>590(^a)</td>
<td>617(^a)</td>
<td>3.9(^a)</td>
<td>0.66(^a)</td>
<td>92 000(^a)</td>
</tr>
<tr>
<td>BODIPY TR</td>
<td>589(^a)</td>
<td>617(^a)</td>
<td>5.4(^a)</td>
<td>NA</td>
<td>68 000(^a)</td>
</tr>
<tr>
<td>Alexa 633</td>
<td>632(^a)</td>
<td>647(^a)</td>
<td>3.2(^a)</td>
<td>NA</td>
<td>159 000(^a)</td>
</tr>
<tr>
<td>Alexa 647</td>
<td>650(^a)</td>
<td>668(^a)</td>
<td>1.0(^b)</td>
<td>0.33(^b)</td>
<td>270 000(^a)</td>
</tr>
<tr>
<td>Cy5</td>
<td>646(^b)</td>
<td>664(^b)</td>
<td>1.0(^b)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(^a\) Data extracted from reference [67].

\(^b\) Data are from www.iss.com/resources/reference/data_table/LifetimeDataFluorophores.html.

NA = Not Available.

The brightness of a fluorophore, which accounts for the quantity of light absorbed as well as the quantum yield, is calculated as the product of the extinction coefficient \( \varepsilon \) and the quantum yield \( \varphi \). This value can be used to make comparisons between different fluorophores [68].

A survey of the fluorophores utilized in all annotated FP assays from the BioAssay Research Database (BARD) provides insight into the probes commonly applied to HTS (figure 8). Table 2 lists information about the properties of these probes. Among the fluorophores, fluorescein dyes are the most common choice, accounting for 47% of the probes and several other assays utilize probes with the same spectral region, such as BODIPY FL and Alexa 488. Fluorescein is a xanthene dye that was first synthesized in the 1870s [69]. It has
a high quantum yield and is widely used in biological research and medicine. Twenty-four percent of the probes were rhodamine dyes, including tetramethylrhodamine (TAMRA). The third most commonly used probe was Cy5 (11%), which has similar excitation/emission spectra to Alexa 647. About half of the probes in figure 8 are red-shifted towards the yellow, orange and red spectrum. Red-shifted probes are preferred for strong affinity interactions that require low concentrations of probe, as their use avoids interference in the blue/green range of library compounds, which are typically present at concentrations much higher than the probe [70]. From table 2, it is apparent that the values for the listed probes are less than 6 ns, which often work well for smaller ligands. Larger ligands require higher values, although not as many of these fluorophores are available. BODIPY probes have slightly higher values than other fluorophores with a comparable spectrum. For example, table 2 shows that BODIPY FL has a lifetime nearly 2 ns longer than either FITC or Alexa 488. Pyrene derivatives have lifetime in the ~20–100 ns range and have been utilized in FP assays, although these fluorophores have emission spectra in the UV range [71, 72]. For very large ligands, metal-ligand complexes with ruthenium (τ ≈ 400–600 ns), osmium (τ ≈ 19 ns) and rhenium (τ ≈ 3000 ns) have been used as probes in FP assays, due to the long lifetimes and high quantum yields [73–77]. Recently, azadioxidaziridinuilenium (ADOTA+), a bright organic dye with red emission (550–600 nm) and a long lifetime (τ ≈ 25 ns) was reported and used to study an ADOTAnitriG-IgG complex by anisotropy [78].

Labeling a molecule with a fluorophore to develop a successful FP assay can be an iterative process. If structural information is available for the molecules of interest, it can serve as a guide for the labeling strategy. Molecular modeling based on structural information has contributed to the design of small molecule-based probes [16, 23, 71, 79–84]. Moreover, information about structure–activity relationships has also enabled the design of labeled small molecules [26]. Upon binding, the fluorophore should ideally be immobile to prevent rotation of the fluorophore, which has been referred to as the ‘propeller effect’ [5]. Flexibility in the attachment of a probe causes depolarization and alters the relationship between polarization and molecular weight, which reduces the assay quality. Therefore, the conjugation chemistry utilized for labeling can impact the assay performance and overall success [85–90]. While local rotational motions of the probe might be avoided by the use of a very short linker, it is possible that such a design will interfere with the ligand binding affinity. In this case, the linker portion might be extended. An example of the substantial effects that probe choice and linker length can have on binding affinity is provided by Cornelius and colleagues, who developed a HTS FP assay to identify modulators of the 5-HT2C receptor [81]. A number of serotonin analogs were synthesized with different probes (Cy3B, TMR, EVOBlue™ 10) and various lengths of aminoalkyl linkers. The affinities of the labeled serotonin analogs to the 5-HT2A, 5-HT2B, and 5-HT2C receptors were determined and compared. The best ligand contained the shortest linkage and the Cy3B probe, whereas low (TMR) or no (EVOBlue™ 10) binding was detected with the same linkage and another probe. When retaining the Cy3B probe, extending the linkage from just 2 to 3 carbons resulted in a 12-fold weaker affinity, which emphasizes that relatively small changes in the linker can have a substantial impact on binding affinity.

Unfortunately, it is difficult to predict whether the characteristics of a fluorophore might affect assay performance due to interference with protein function, ligand affinity or other mechanisms. As an example, some researchers have reported more interference with binding affinity by fluorescein labels than by BODIPY [91, 92]. However, the opposite has also been observed. In optimizing a FP assay based on the interaction between a labeled peptide fragment from the redox sensor protein, Keap1, and the transcription factor, Nrf2, Inoyama and colleagues examined a variety of peptide ligands [16]. After choosing an optimized peptide ligand, four different fluorophores (Fluorescein, BODIPY-FL, Cy3B and Cy5) were compared with respect to the dynamic range, assay quality and binding affinities. The Cy3B probe enabled the largest dynamic range (Δ167.7 mA), highest Z’ value (0.95) and strongest affinity (Kd = 6 nM), whereas the BODIPY-FL probe resulted in the lowest dynamic range (Δ66.6 mA), a reduced Z’ value (0.86) and a weaker affinity (Kd = 20 nM). In a more extreme example, we evaluated the performance of several fluorophores during the development of FP assays to identify inhibitors of the interaction between the G-protein alpha subunit, Go13, and a fluorophore-labeled BGS12 GoLoco polypeptide [93]. The assay was initially developed and miniaturized with a FITC labeled GoLoco polypeptide (figure 9, green squares). To reduce the interference by library compounds, attempts were made to develop the assay with red-shifted fluorophores. While replacement of the FITC label with TAMRA improved the dynamic range of the assay (figure 9, black circles), no binding was observed with BODIPY Texas Red (figure 9, red triangles). The loss of binding associated with BODIPY Texas Red was attributed to the hydrophobicity of the probe interfering with the interaction and/or inducing aggregation of the probe. Together, these examples demonstrate the value in examining several fluorophores during assay optimization.

### 4.2. Optimization of assay conditions

If an assay has already been established for a target of interest, such as an enzyme or molecular interaction, the established buffer can serve as a starting point for further assay development. Nevertheless, it is worth examining how the solution conditions affect the FP assay performance. According to equation (1), temperature contributes to the observed FP and can also
impact the fluorescence lifetime of some probes, such as coumarines and cyanine dyes [94, 95]. In reality, it is often impractical to vary the temperature of FP assays for HTS, but it is important to maintain a consistent temperature for reproducibility. Modifications to the buffer conditions, including pH and type of buffering compound, can result in substantial improvements to the dynamic range of an assay. Not only do the solution conditions influence binding affinity and enzymatic activity, they can also alter the properties of a probe. For instance, the spectral properties (fluorescence lifetime, quantum yield and extinction coefficient) of some probes vary as a function of pH [5]. In the case of the very popular fluorophore, fluorescein, the dye’s fluorescence intensity undergoes a dramatic decrease upon lowering the assay pH below approximately 7.0 due to fluorescein’s specific pKa value. Also, the electrostatic properties of ligand binding sites can alter the fluorescence lifetime of a probe [96].

The influence of salt concentration and the presence of divalent cations should be examined during assay development. As described above, very strong affinity interactions ($K_d < 1 \text{nM}$) require the use of low probe concentrations, which reduces the signal intensity and thus increases the background noise. In this case, it might be possible to increase the ionic strength of the solution to weaken the affinity to a range that will allow a higher probe concentration and thus improve the assay performance [97]. Other additives, such as reducing agents, chelators and albumin should be considered. Finally, the addition of a detergent, such as Brij-35, NP-40, Triton X-100 or Tween 20 at a concentration of ~0.01% can improve assays by reducing protein adsorption to the surfaces of the wells. Although systematically examining numerous solution conditions is laborious, the efforts can be rewarding. As an example, Zhai and colleagues reported that assay buffer optimizations increased the assay signal window by 30%, which allowed a 33-fold

Figure 9. Probe choice influences FP assay performance. A FP assay was developed to report on the interaction between $\alpha_{G_\text{i1}}$ and fluorophore labeled RGS12 GoLoco polypeptide. While peptides labeled with both TAMRA (15 nM, black circles) and FITC (10 nM, green squares) fluorophores enabled the development of robust assays, binding was not observed with BODIPY Texas Red (BTR) (50 nM, red triangles). The data were collected with a ViewLux Microplate Imager (PerkinElmer) from 4 $\mu$l reactions in a 1536-well format with different settings for TAMRA (Light Energy: 50 000, Time: 95 s, G-factor: 0.98, Binning: 2X and $\lambda_{EX} = 525 \text{nm}$/$\lambda_{EM} = 598 \text{nm}$) and FITC (Light Energy: 50 000, Time: 70 s, G-factor: 1.0, Binning: 1X and $\lambda_{EX} = 480 \text{nm}$/$\lambda_{EM} = 540 \text{nm}$).

Figure 10. Absorption and emission spectra for dipyridamole. (a) Absorbance spectrum of 100 $\mu$M dipyridamole in PBS pH 7.4 (blue solid line) shows two excitation peaks at 295 nm and 415 nm (absorbance of buffer only is shown as a red dashed line). Data were collected with an Agilent 8453 G1103A UV-Vis Spectrophotometer. (b) Emission spectra collected on a Tecan Safire2 with a 384-well format for a solution of 4 $\mu$M AGP, 0.4 $\mu$M dipyridamole, 0.01% Tween 20, PBS, pH 7.4 after excitation at 295 nm (red dashed line) and 415 nm (blue solid line). The emission spectra of the buffer at 295 nm (purple dotted line) and 415 nm (green dot-dash line) is also shown. Exciting the sample at 415 nm provides a two-fold higher signal over excitation at 295 nm.
reduction in the required protein concentration and an improvement in the signal stability from 15 min to 24 h [22]. Both signal stability and the time required to achieve maximal signal must be examined during optimization. The amount of time required for equilibrium binding of a labeled probe can vary among assays and should be incorporated into the optimized protocol. For example, during assay optimization, Zhai and colleagues observed time-dependent binding of Bfl-1 to the fluorescent probe. Therefore, the authors included a 1 h incubation of Bfl-1 in the presence of compounds before adding the FITC-BH3 peptide, which was allowed to incubate for an additional 4 h before a measurement was made [22]. Similarly, Rohe and colleagues reported that their kinase tracer required at least 2 h to bind Myt1 kinase, whereas the binding process was complete in 5–10 min for the kinases Abl1 and Btk [23]. At our center, we routinely monitor the FP signal for at least 24 h.

Another important consideration is whether the excitation/emission spectra are optimal for a given probe under the particular assay conditions [5]. As described above, we miniaturized a FP assay to identify compounds that bind to α1-acid-glycoprotein (AGP), which is one of the major plasma proteins responsible for drug binding [46]. Initially, we miniaturized the assay based on previously reported methods for displacement of the fluorescent probe, dipyridamole, which indicated $\lambda_{\text{EX}} = 340$ nm and $\lambda_{\text{EM}} = 530$ nm [45]. However, in 1536-well format the assay yielded a small dynamic range of only ~40 mP and significant variability among replicates. Therefore, we measured the excitation spectra of dipyridamole and observed two excitation peaks at ~295 nm and ~415 nm (figure 10(a)). The absorbance of dipyridamole was low at 340 nm, which explained our poor assay results. Excitation of dipyridamole at $\lambda_{\text{EX}} = 415$ nm resulted in an emission peak ~2 times greater than $\lambda_{\text{EX}} = 295$ nm (figure 10(b)). The dynamic range of the assay was dramatically improved by switching to an excitation wavelength of 405 nm, which was accessible by our plate readers (figure 11).

### 5. Flagging assay interference compounds from HTS data

As with any other technique, FP also has limitations, such as the potential for a probe to interfere with the biological system under investigation, difficulties in studying weak-affinity interactions and interferences by compounds that cause light scattering. However, one advantage of FP is that, due to the ratiometric nature of the fluorescence polarization measurement, there is less interference by compounds with overlapping spectra [65]. Equations (3) and (4) show that the values of FP and FA do not depend on the absolute intensities of the emission light from the parallel and perpendicular channels and are therefore independent of fluorophore concentration (within the limits of instrument linearity and sensitivity). Deviations from this relationship, such as a positive correlation between fluorescent probe concentration and observed FP, might be indicative of probe aggregation [6]. Interferences due to the absorption of light or color quenching often have minimal influence on FP values because the interference contribution (C) is proportional among the $I_1$ and $I_\perp$ channels (13). Nevertheless, proportional interferences do increase the experimental noise, which reduces the precision of FP measurements [65]. On the other hand, additive contributions to interference, such as background fluorescence, do alter the FP measurement as shown by (14). It is difficult to correct the interference caused by auto-fluorescent compounds.

$$\text{FP} = \frac{C I_{\perp} - I_{\parallel}}{C I_{\parallel} + C I_{\perp}} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$  \hspace{0.5cm} (13)$$

$$\text{FP} = \frac{(C + I_{\parallel}) - (C + I_{\perp})}{(C + I_{\parallel}) + (C + I_{\perp})} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$  \hspace{0.5cm} (14)
Fluorescence spectroscopic profiling of more than 70,000 library compounds indicated that a significant percentage of the compounds are capable of interfering with fluorescent assay readouts, which was around 5% when using blue-shifted fluorophores ($\lambda_{\text{EX}} \approx 360\,\text{nm}/\lambda_{\text{EM}} \approx 450\,\text{nm}$) and nearly 0.2% with fluorophores in the fluorescein region ($\lambda_{\text{EX}} \approx 480\,\text{nm}/\lambda_{\text{EM}} \approx 520\,\text{nm}$). Switching to red-shifted fluorophores ($\lambda_{\text{EX}} \approx 525\,\text{nm}/\lambda_{\text{EM}} \approx 598\,\text{nm}$) reduced the percentage of interference compounds to about 0.01% [70]. Typically, fluorescence interference is due to spectral overlap with a library compound, but it can also result from impurities, precipitation and aggregation. Absorption of light by a compound reduces the fluorescence intensity (FI) measured (figure 12(a), blue circles), whereas fluorescent compounds with a spectra that overlaps that of the probe increase the FI measured (figure 12(b)). Compounds that act as chemical quenchers often reduce the observed FI in a concentration dependent manner (figure 12(a), black squares). In a process known as static quenching, compounds can bind the probe directly and cause a loss of fluorescence. Quenching compounds can also interact with the excited state of the probe to prevent fluorescence emission, which is called dynamic quenching [98]. Additionally, quenching compounds can induce aggregation of the probe by engaging in pi-stacking interactions between hydrophobic regions of probe and compound. Compounds with poor solubility form precipitates that can cause light scattering, which can interfere by several mechanisms. Intense scattered light can increase both the observed FI and FP values.

Figure 12. Examples of artifacts from concentration-response screening data of a FP assay with a BODIPY Texas Red probe. (a) The dye Evans Blue (blue circles) reduces FI by absorbing the emission from the BODIPY Texas Red probe. The compound GW5074 (black square) likely induces aggregation to cause a concentration dependent reduction in FI. (b) D&C Red Dye 27 has a spectra that overlaps with the probe and displays a dose dependent increase in FI. The data were collected with a ViewLux Microplate Imager (PerkinElmer) from 2.5 $\mu l$ reactions in a 1536-well format with the following settings: Light Energy: 30,000, Time: 10 s, G-factor: 0.827, Binning: 2X and $\lambda_{\text{EX}} = 525\,\text{nm}/\lambda_{\text{EM}} = 598\,\text{nm}$.

Figure 13. Distribution of $\Delta$FI values among 1527 hits from a concentration-response HTS screen. Total fluorescence intensity was calculated with the data from each compound at all concentrations using equation (15) and the appropriate values were applied to equation (16). Plotting the data reveals a Gaussian distribution where $\Delta$FI > 3$\sigma$ of zero are likely to be interference compounds.
However, light scattering can also reduce the amount of excitation and emission light within the optical path. The use of red-shifted probes reduces interference by light scattering due to the decrease in intensity of scattered light at longer wavelengths [99].

To identify interference compounds within raw FP data from concentration–response screening formats, the total FI is first calculated with the data from all concentrations of a given compound using (15).

\[
FI = I_h + 2I_L
\]  

Next, the largest (FI_{max}) and smallest (FI_{min}) values are identified and applied to (16) to obtain \(\Delta FI\).

\[
\Delta FI = \frac{FI_{max} - FI_{min}}{FI_{max}}
\]

Plotting the values of \(\Delta FI\) from qHTS should result in a normal distribution similar to figure 13. Compounds with a \(\Delta FI\) less than \(3\sigma\) are considered to be in the normal range, whereas those with a \(\Delta FI\) greater than \(3\sigma\) are flagged as putative interference compounds. Depending on the number of hit compounds, two types of filters might be applied to the data. Tier 1 will include those compounds with values beyond \(6\sigma\); whereas, tier 2 will comprise compounds with values between \(3\sigma\) and \(6\sigma\). While this method will identify interference compounds, it is also possible to minimize the impact of background fluorescence by making a measurement after library compounds are introduced, but before addition of the probe. The background fluorescence values are subsequently subtracted from the final measurements. This method works well provided that the measured background fluorescence is in the linear range of detection.

Another way to identify interference compounds is to determine whether the polarization values of the hit compounds fall outside of the range defined by the FP values for the low and high controls [93]. First, the raw data from each plate is normalized so that the average FP values for the low control are set to 0% and the average FP values for the high control are set to 100%. Following the normalization, compounds that result in FP values \(5\sigma\) above 100% are excluded due to interference, likely by inducing aggregation of the probe or substrate. Similarly, compounds that give FP values \(5\sigma\) below the 0% control are excluded due to interference with the probe fluorescence.

In addition to the strategies described above, cheminformatic filters should be applied to the list of actives to flag problematic compounds. The pan assay interference compounds (PAINS) filters described by Baell et al can recognize compounds that are likely to cause assay interference [100]. The estimated hit rate for PAINS compounds is in excess of the typical 0.5–2% hit rate of HTS, which might prevent the identification of compounds with genuine activity against the target. The PAINS filters can be applied using the cheminformatics suites from Tripos or Schrödinger as well as the open-source software, KNIME or the FAF-Drugs3 server [101–104].

The ‘HTS filter’ within the cheminformatics component of Pipeline Pilot (BIOVIA) is also able to identify problematic reactive compounds. Another useful filter, the Rapid Elimination of Swill (REOS) was designed to identify compounds likely to cause assay interference as well as strong electrophiles that irreversibly bind to assay components or proteins [105]. Counter assays can also be performed to distinguish reactive compounds among hits identified from a screen [106].

6. Conclusion

Since the development of microplate readers capable of FP measurements in the 1990s, FP assays have become widely utilized in HTS and drug discovery. In addition to the convenient homogeneous format, optimized FP assays are often robust with an excellent dynamic range and can be further miniaturized for ultra-high-throughput screening. The application of FP assays to increasingly complex biological processes has been driven by advancements in assay design, fluorescent probes and technology. The development of related FP assays that incorporate different types of fluorophores, such as fluorescein and rhodamine, aids in the selection of an optimal ligand and provides a counterscreen to identify some types of interference compounds. The utility of FP assays in HTS is underscored by the diversity of biological systems successfully targeted by this approach.

Acknowledgments

This work was supported by the NCATS Division of Pre-Clinical Innovation Intramural Program. We thank Dr R MacArthur for the critical reading of this manuscript.

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