Development and Application of Fluorescence Polarization Assays in Drug Discovery

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Abstract: Fluorescence polarization technology has been used in basic research and commercial diagnostic assays for many decades, but has begun to be widely used in drug discovery only in the past six years. Originally, FP assays for drug discovery were developed for single-tube analytical instruments, but the technology was rapidly converted to high-throughput screening assays when commercial plate readers with equivalent sensitivity became available. This review will discuss fluorescence polarization assays in current use in drug discovery research as well as those in development that will likely be used in the near future. These assays include targets such as kinases, phosphatases, proteases, G-protein coupled receptors, and nuclear receptors.

INTRODUCTION

Early drug discovery is a challenging and expensive process beginning with target identification and validation and culminating years later with compounds ready for pre-clinical trials. Many discovery projects utilize fluorescent biochemical assays for screening compound libraries, for profiling compounds, and for generating data for determining structure-activity relationships (SAR). In the past eight years, screening methods have improved so dramatically that many companies no longer use tubes or 96-well plates, but rather routinely conduct ultra-high-throughput screening, performing 100,000 assays in 24 hours. Although the 384-well plate is now the standard format, within a few years, the standard will progress to the 1536-well plate. These changes have created a new set of challenges in early drug discovery, such as creating inexpensive, miniaturized, homogeneous fluorescent assays that can be universally applied to an entire drug target family and developed quickly. Fluorescence polarization (FP) has successfully surmounted many of these challenges; nearly all major pharma and biotech companies now use FP as one of the standard detection technologies.

FLUORESCENCE POLARIZATION DESCRIPTION AND THEORY

FP theory arises from the observation that when a fluorescently labeled molecule is excited with plane polarized light, it emits light that has a degree of polarization that is inversely proportional to its molecular rotation. Large fluorescently labeled molecules remain relatively stationary during the excited state (about 4 ns in the case of a fluorescein label) and the polarization of the light remains relatively constant between excitation and emission. Small fluorescently labeled molecules rotate rapidly during the excited state, however, and the polarization of the light changes significantly between excitation and emission. Therefore, small molecules have low polarization values and large molecules have high polarization values. For example, a fluorescein-labeled phosphopeptide has a relatively low polarization value, but when bound to a very large protein, the complex has a high polarization value.

Fluorescence polarization is a versatile technique for measuring equilibrium binding, nucleic acid hybridization, and enzymatic activity. Fluorescence polarization assays are homogeneous in that they do not require a separation step and do not require attachment to an immobilized phase. Polarization values can be measured repeatedly and after the addition of reagents, because measuring the polarization is rapid and does not destroy the sample. Generally, this technique can be used to measure polarization values of fluorophores from low picomolar to micromolar levels. Polarization does not change with fluorescence intensity, therefore a 100 pM and a 500 nM solution of fluorescein should have the same polarization values.

For high-throughput screening assays, fluorescence polarization can be used to directly measure binding and dissociation between two molecules if one of the binding partners is small and fluorescently tagged. The binding can be monitored simply by reading the polarization value of the small fluorescent molecule before and after it binds to a larger molecule. Developing competitive binding assays, which can be used to measure analyte concentrations directly in solution, is straightforward. The diagnostic market has used this technique widely for more than 20 years; the drug screening market has recently adopted it. Table 1 presents many FP-based assays that are or could be used in drug discovery assays. The wide variety of assays shown in the table demonstrates the versatility of the FP technique and how it could be used in many steps throughout the discovery process.

Fluorescence anisotropy (commonly noted as r or sometimes A) is another way to describe how the plane of polarized light changes between excitation and emission with a rotating fluorophore. As both polarization and anisotropy reveal the same information, using one method rather than the other is purely a matter of convenience. However, given a mixture of different fluorescent molecules, different equations are used to calculate each set of

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Table 1. Selected Applications of FP Assays in Drug Discovery

Target and Comment	References
70S ribosome-E.coli/fluorescent antibiotic binding. Large 1536 well screen and data analysis	[14]
Adenine transferase, 1536 well HTS direct incorporation assay	[53]
AKT serine/threonine kinase assay	[40]
angiotensin-converting enzyme binding to fluorescent inhibitors	[54]
cAMP binding to Gs-coupled receptors	[55]
cAMP FPIA	[52]
Capillary electrophoresis coupled to FP for antibody binding and protein/DNA	[56,57]
Carbohydrate/lectin binding	[58,59]
cholecystokinin (CCK) receptor	[60]
Cyclin-dependent kinase 2/cyclin E interaction with peptide inhibitor, 1536 well HTS	[61]
DNA detection with fluorescent nucleotides or oligonucleotides	[62-68]
Estrogen receptor fluorescent ligand binding	[21,26,43,69]
Genotoxicity testing measuring expressed GPP FP to to avoid autofluorescence intensity	[70]
Glucocorticoid receptor/fluorescent ligand binding	[19][1]
GPCR ligand binding	[50-52,55,71-73]
Green fluorescent protein	[56,70,74]
Isoleucyl-tRNA synthetase	[75]
Kinase activity (tyrosine and serine/threonine)	[43]
Kinase activity measurement using phosphopeptide detection using cationic polyamino acids	[44]
Kinase incorporates thiol, which is biotinylated detected by FP	[46]
Kinase, phosphatase, protease, FP detection using polyarginine	[45]
P53-DM2 interactions	[70]
Peptide cleavage by viral proteases	[76]
Peptides that mimic glycosaminoglycans: high-affinity ligands for a hyaluronan binding domain.	[77]
Peptidyl-tRNA hydrolase cleaveage of fluorescent Lys-tRNA^{lys}.	[78]
Phosphatase detection	[43]
Protein Interactions p53/DM2 binding inhibition by peptides	[79]
Protein kinase C assay	[80]
Protein/DNA binding (CBP binding to phosphorylated CREB-DNA complex)	[2]
Protein/DNA binding (TATA box binding protein binding to TATA box DNA)	[81]
Ribozyme activity measurement	[82]
SNP detection	[68,83,84]
Tyrosine kinase activity	[42,85]
Review Articles	[1-3,5-8,14,18]

molecules's fractional contribution to the overall emission intensity and thus the polarization and anisotropy values. Applying these equations incorrectly can introduce a small error. The possible error is described in a recent review [1]. The theory, practice, and references for fluorescence polarization and anisotropy have been covered in several review articles and therefore will be covered here only to provide basic understanding of how the technique has been applied to drug discovery [2-7].

[1] A complete commercially available assay (PanVera LLC, Madison, WI)

Fluorescence polarization (P) and anisotropy (r) are defined as:

$$\text{Polarization} = P = \frac{I_{Vertical} - I_{Horizontal}}{I_{Vertical} + I_{Horizontal}}$$

and

$$\text{Anisotropy} = r = \frac{I_{Vertical} - I_{Horizontal}}{I_{Vertical} + 2 * I_{Horizontal}}$$

Where $I_{Vertical}$ is the intensity of the emission light parallel to the excitation light plane and $I_{Horizontal}$ is the intensity of the emission light perpendicular to the excitation light plane. *P* and *r*, being ratios of light intensities, are dimensionless. Experimental data are often expressed in millipolarization units (1 Polarization Unit = 1000 mP Units) or millianisotropy units. (1 Anisotropy Unit = 1000 mA Units).

The formulae to interconvert polarization and anisotropy are:

$$P = \frac{3r}{(2 + r)} \quad \text{and} \quad r = \frac{2P}{(3 - P)}$$

Fundamentally, polarization is a relationship of fluorescence lifetime and how fast the fluorophore rotates in the time between the excitation and the emission. The principal factors controlling rotation are molar volume (*V*), the absolute temperature (*T*), and viscosity (η), but the literature does not always describe the relationship between these clearly. For clarification, the derivation of the rotational correlation time (θ) and the rotational relaxation time (ρ_o) from the work of Perrin and Weber are described in two recent reviews by Owicki and Jameson, *et al.* respectively[1,8]. The rotational correlation time is taken from the Perrin equation:

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_o} - \frac{1}{3}\right) * \left(1 + \frac{\tau}{\theta}\right)$$

and is defined as:

Rotational Correlation Time

$$(\theta) = \frac{\eta V}{RT}$$

As described in the work of Jameson [8], the rotational relaxation time is taken from the Perrin/Weber equation which was first described in 1926 [9].

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_o} - \frac{1}{3}\right) * \left(1 + \frac{3\tau}{\rho}\right)$$

and is defined as:

Rotational Relaxation Time

$$(\rho_o) = \frac{3\eta V}{RT}$$

R is the gas constant, τ is the fluorescence lifetime, P is the polarization, and P_0 is the limiting polarization.

In either case, if the lifetime, viscosity, and temperature are held constant, then the molecular volume (and thus the polarization or anisotropy) determines the rotation. The larger the volume, the slower the molecule rotates and the higher the polarization and anisotropy values. Note that the reported rotational relaxation time will always be three times longer than the rotational correlation time.

If a fluorophore has a very short fluorescence lifetime, then the starting polarization will generally be too high to use in a screening assay. In this situation, even a small molecule, such as a labeled steroid or peptide, simply does not have enough time to rotate during the fluorescence lifetime and depolarize the emission relative to the excitation light. Because the limiting polarization (≈ 500 mP, ≈ 400 mA) does not change with lifetime when the fluorophore is excited near its maximal value, choosing a very fast dye will limit the range of the assay. Fluorophores with long lifetimes (>100 ns) are more suitable for large molecules [10,11], but often possess some characteristics undesirable for high-throughput screening. These undesirable traits can include excitation in the ultraviolet range, which leads to significant compound interference or low quantum yields, which mean the fluorophore cannot be detected at a low nanomolar concentration. The relationship between fluorescence lifetime and polarization values in protein-protein interactions has recently been discussed [8].

A recent review summarizes the choice of fluorophores for labeling in FP experiments [1]. Additionally, the type of linker connecting the fluorophore to the tracer molecule can strongly affect the degree of shift in polarization when a larger molecule binds a small fluorescent tracer. In one example, DNA is labeled with DTAF (diaminotriazinylamino-fluorescein) to monitor hybridization by FP [12]. DTAF contains a six-carbon ring structure that separates the fluorescein and the nucleic acid and prevents the fluorescein from spinning (the propeller effect) relative to the nucleic acid. When hybridization occurs, a much larger shift in polarization appears than with standard fluorescein labeling techniques. In a second example, a fluorescein arsenical hairpin binder (FlAsH™) [13], a biarsenic derivative of fluorescein, labels proteins by binding to a specific tetracysteine motif containing the amino acid sequence Cys-Cys-Xaa-Xaa-Cys-Cys. This unique tetracysteine motif can be incorporated within protein sequences, enabling site-specific protein labeling. Because FlAsH has two points of attachment for binding to its target, FlAsH is more rigidly linked to the labeled target and thus more sensitive than standard labeling techniques in FP measurements (manuscript in preparation, PanVera LLC). This feature generates greater shifts in polarization when measuring direct protein interactions by FP.

Instrumentation performance is perhaps the most important factor in an FP assay, in terms of both measuring with high accuracy and precision and dealing with common interferences. These interferences in FP are described in a review by Owicki [1]; specific techniques for handling background signal in a 1536-well format high-throughput screen are described in great detail in a review by Turconi [14].

Table **1** demonstrates that high-precision FP measurements enable achieving very high Z'-factors [15], even with very low total shifts in polarization. For example, even if an assay yields a total shift of only 30mP, if the

Table 2. High Precision FP Measurements Effect on the Z' Factor. Two 10-Point Competition Curves were Generated Using PanVera's Tyrosine Kinase-Green HTS kit. The Phosphopeptide Tracer was Bound to Different Amounts of Antibody and Competed off Using an Unlabeled Phosphopeptide. After Reaching Equilibrium, Each Data Point was Measured Six Times on an Affinity FP Reader Supplied by CRI

mP Range in Curve	Ave. Standard Deviation (mP)	Z' Calculated from Data	Theoretical Z' if Ave. Standard Deviation was 4.0 mP
42.3 mp -272.4 mP	1.12	0.97	0.40
37.5 mP-64.6 mP	0.75	0.84	0.11

standard deviation on the measurement is <1.0 mP, the Z' is still 0.8. In contrast, if the standard deviation in the same assay is 5 mP, then Z' is 0. These results also suggest that selecting complexes with the tracer only 30–40% bound, rather than 80% bound, could produce significant reagent cost savings in some experiments.

DRUG DISCOVERY USING FLUORESCENCE POLARIZATION

One attractive aspect of FP assays is that developing and validating an HTS assay is relatively straightforward once the analytical assay has been developed and validated. Table **2** contains a list of assays (with references) that either have been used in high-throughput screening or could be useful in screening or compound profiling. Some applications in which FP has been used extensively in drug discovery are described in greater detail below.

NUCLEAR RECEPTORS

A superfamily of ligand-activated transcription factors, commonly called the nuclear receptors, regulate the expression of genes fundamental for growth, differentiation, metabolism and reproduction. Because nuclear receptors are activated by small, specific ligands and play critical roles in many human pathologies, they are highly 'druggable' targets, and thus represent an area of intense pharmaceutical research. As one example, estrogen receptor is a key drug target in breast cancer, osteoporosis, and cardiovascular disease drug discovery. Simple, rapid FP-based assays have been developed for screening compound libraries to identify drug molecules that modulate nuclear receptor function.

Though diverse in the biological functions they control, nuclear receptors share common functional domains including the well-conserved DNA binding domain (DBD), a less-conserved hinge area, and the C-terminal ligand binding domain (LBD). The LBD, in addition to providing a binding pocket for specific endogenous ligands such as the classical steroid hormones, some vitamins, and metabolic intermediates, contains surfaces which interact with coregulator proteins. In addition, the DBD in nuclear receptors binds specific DNA sequences, called response elements that lie within the promoter regions of responsive genes. Binding to an agonist ligand causes a conformational change in the LBD that leads to coactivator protein binding that then enables transcriptional activation of the target gene. FP technology has been used to monitor all three receptor biomolecular binding events: response element [16-18], ligand [19-21], and coregulator interactions ([22], Mary Ozers, PanVera LLC, manuscript in preparation).

RECEPTOR/DNA EQUILIBRIUM BINDING

Nuclear receptor/ response element binding has been studied using methods such as gel-shift assays and transcriptional activation assay. While useful, these methods do not provide a simple way to quantitatively measure specific binding. FP, however, provides a rapid nonradioactive method for measuring these interactions. Several groups have used this technique to measure estrogen receptor/estrogen response element (ERE) binding [18,23,24]. In a standard experiment, a double-stranded fluorescein-labeled oligonucleotide, containing both an estrogen response element and its flanking sequence, is added to increasing concentrations of purified estrogen receptor [Fig. (**1**)]. To demonstrate binding specificity to a response element, adjusting ionic conditions or adding an appropriate excess of nonspecific DNA may be necessary.

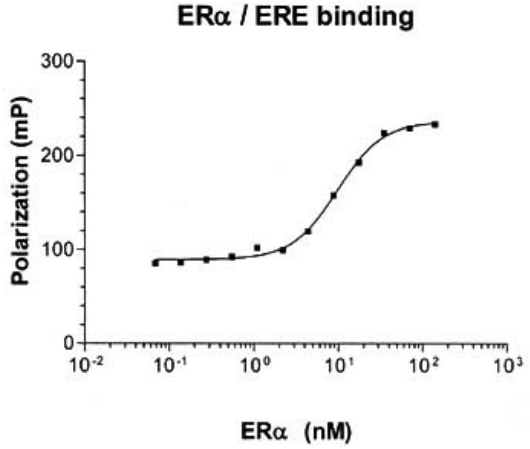

Fig. (1). ER / ERE binding
ERα was serially diluted in ER Binding Buffer containing 25 mM Tris-HCl, pH 7.5, 100 mM KCl, 100 ug/ml BSA, 2 mM DTT, 0.1 mM EDTA and 10% glycerol. Fluorescein-labeled ERE (ERE-f) was added to each tube to a final concentration of 1 nM. The polarization value of the protein/DNA complex was measured using the Beacon ^{TM} Fluorescence Polarization System. At low protein concentrations, most of the ERE-f remained free and so the polarization values were low. As increasing amounts of ER were added, more ERE-f was bound, decreasing its rotation and resulting in a higher polarization value. Nonlinear regression analysis was conducted using Prism GraphPad software. The observed EC_{50} value is 9.7 nM.

Ligand-dependent changes in estrogen receptor/response element binding have been demonstrated with FP, suggesting that conformational changes within the LBD affect the binding properties of the DNA binding domain [25].

LIGAND DISPLACEMENT ASSAYS

Historically, ligand activation of nuclear receptors has been investigated in animal and cell-based studies. Such methods are expensive, time-consuming, and, due to the complexity of the systems, leave open the possibility that the affected gene(s) is not necessarily the targeted gene. Molecular ligand displacement assays overcome some of these inherent problems and are more amenable to HTS. Radio-ligand biochemical assays such as the scintillation proximity assays (SPA, Amersham Pharmacia Biotech, Piscataway, NJ) or FlashPlate assays (PerkinElmer Life Science, Boston, MA) require the receptor and scintillant to be bound to a solid-phase, resulting in potentially disadvantageous conformational changes of the receptor. FP has proved to be a useful format for high-throughput ligand displacement assays, largely due to the small molecular size of nuclear receptor ligands and the ligand/receptor binding affinities being strong. In addition to being simple 'mix and read' assays, FP competitive displacement assays approach the sensitivity of classical radioligand binding assays. Such assays have been developed for screening glucocorticoid receptor [19], estrogen receptor [20,21,26] , progesterone receptor, and androgen receptor ligands.

The two essential components of FP-based nuclear receptor ligand binding assays are a fluorescently conjugated ligand (tracer) and a nuclear receptor protein. The fluorescent ligands typically have a molecular mass of less than 1 kDa. When excited by plane polarized light, these small molecules will tumble freely in solution and emit light that is largely depolarized. However, when bound to nuclear receptor proteins (molecular weight typically 50–100 kDa), the tracer will rotate much more slowly and therefore emit light that remains largely polarized. As shown in the equilibrium binding curve in [Fig. (**2**)], measuring tracer polarizations over a range of receptor concentrations enables one to easily detect receptor/tracer binding and calculate the EC_{50} of that interaction.

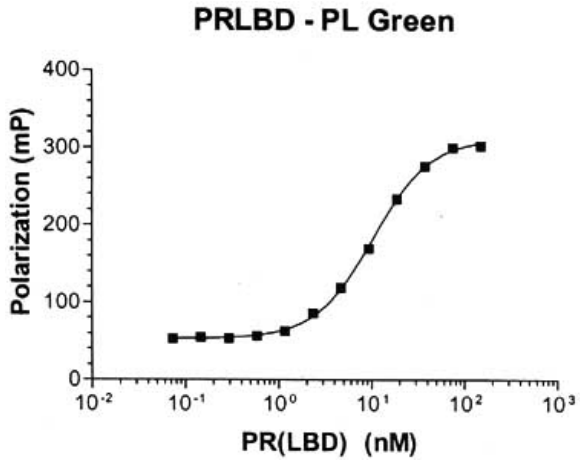

Fig. (2). PRLBD / PL green equilibrium binding.
Serial dilutions of a purified fusion protein of progesterone receptor ligand binding domain PR(LBD) were prepared in microtiter plates. The fluorescently labeled progesterone, PL Green, was added to each well at a final concentration of 1 nM. Following a 2-hour incubation at room temperature, the polarization value of each well was read with the TECAN Polarian, using standard fluorescein filters (485 nm excitation; 535 nm emission). The data presented are averages of triplicate assays. Nonlinear regression analysis was conducted using Prism GraphPad software. The EC_{50} of PRLBD for PL-Red was 10.4nM, +/- 0.9nM.

In the competitive displacement assay, the concentrations of receptor and fluorescent ligand are held constant. Under typical experimental conditions, sufficient receptor to bind 50–80% of the fluorescent tracer is provided to allow for maximal signal/background ratio and for minimal loss of sensitivity. Because most FP-capable plate readers can reliably calculate FP values from 1 nM fluorescent tracer, 1 nM is the tracer concentration frequently chosen. As an example, in an equilibrium binding assay, the glucocorticoid receptor (GR) binds the fluorescent ligand GS2 with a dissociation constant of 0.5 nM. The conditions chosen for the competitive displacement assay were 1 nM GS2 and 4 nM GR, because 4 nM GR will bind approximately 80%, or 0.8 nM, of GS2. By adding test compounds to this complex and measuring the resultant changes in polarization values, the effect of each test compound can be assessed. In HTS primary screens, single concentrations of test compounds are generally assayed first to identify 'hits', that is, compounds that disrupt the GR/GS2 complex and, thus, produce decreased polarization values. In HTS secondary screens, promising competitor compounds can be tested further over a range of concentrations in FP assays to calculate IC_{50} values [Fig. (**3**)].

Traditionally, the affinity of an inhibitor for a receptor (K_i) has been calculated from its observed IC_{50} using the Cheng and Prussoff equation:

$$K_i = \frac{IC_{50}}{\left(1 + \frac{L}{K_d}\right)}$$

where IC_{50} is the concentration of inhibitor required to displace 50% of the labeled ligand, L is the total concentration of labeled ligand and K_d is the dissociation constant for the labeled ligand [27]. However, this equation was derived in a context in which the concentration of the labeled ligand, L, approximates its free (not total) concentration and the IC_{50} implies the free concentration of inhibitor that reduces binding of the labeled ligand by 50%. In FP, unlike conventional radioligand binding experiments, concentrations of total and free ligands typically do not approximate each other and receptor concentrations tend to be high. When the concentrations of total receptor and bound receptor/ligand are known, Kenakin [28] offers the following solution:

$$K_i = \frac{(B * I * K_d)}{(L_r * R_T) + B * (-R_T - L_T + B - K_d)}$$

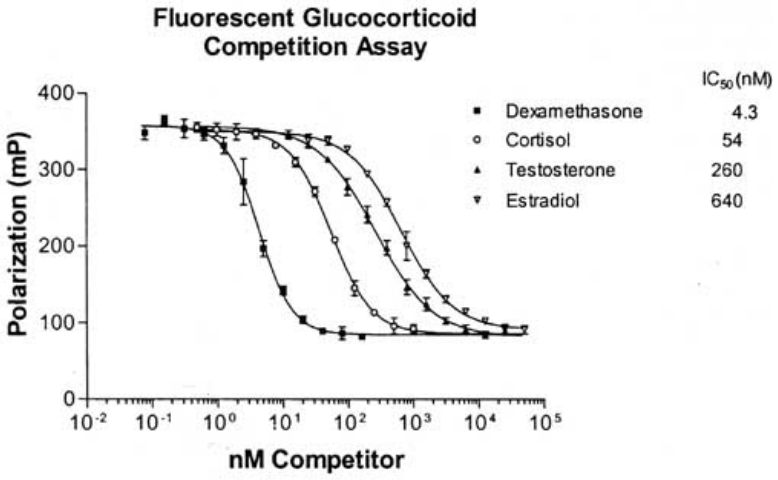

Fig. (3). GR/GS red competition.

Serial dilutions of ligands were prepared in microtiter plates. Glucocorticoid Receptor (GR) and a rhodamine conjugated GR ligand (GS Red) were added to each well. Following a two hour room temperature incubation period, fluorescence polarization measurements were taken using the TECAN Polarian, using standard rhodamine filters (535 nm excitation; 590 nm emission). Representative experiments of 3 replicates are shown. Nonlinear regression analysis was conducted using Prism GraphPad software. IC_{50} values are listed.

Where B equals the concentration of bound labeled ligand, I equals the inhibitor concentration at which 50% of the labeled ligand is bound, R_T is the total concentration of receptor, and L_T is the total concentration of labeled ligand. Note that when using the IC_{50} to estimate the K_i, any errors caused by high receptor and ligand concentrations will lead to overestimations of the K_i. For example, if the receptor concentration is 10-fold greater than the K_d, the IC_{50} will overestimate the K_i by about 10-fold. Table (**3**) presents some observed IC_{50} values and the calculated K_i values using both the Cheng and Prussoff equation and the more appropriate Kenakin equation.

Table 3. Glucocorticoid Receptor Competition Assay with Fluorescent Glucorticoid (GS1). Total Receptor Concentration was 4 nM; Total GS1 Concentration was 1 nM. Calculation of K_i from IC_{50} Using Models of Cheng-Prusoff or Kenakin. All Units are nM

Ligand	IC_{50}	K_i (Cheng)	K_i (Kenakin)
Dexamethasone	11	4	1.1
Beclomethasone monopropionate	7	2	0.70
Cortisol	31	10	3.2
Fluocinonide	41	14	4.2
Prednisone	670	223	68
Testosterone	1230	410	124
Estradiol	1910	637	195

RECEPTOR/COACTIVATOR BINDING

Ligand-directed coactivator proteins modulate transcriptional activation by nuclear receptors. When an agonist ligand binds to the LBD, a hydrophobic binding surface found within helix 12 of the LBD becomes exposed and binds to the coactivator protein. Upon formation of this complex, other transcriptional machinery will assemble and transcription of downstream genes will occur. On the other hand, when an antagonist ligand binds to the LBD, the binding surface within helix 12 remains inaccessible to coactivators, so transcription will not proceed. A critical need in the drug discovery process is to identify and classify ligands as agonist or antagonist as early as possible. This need is particularly crucial in the area of orphan receptors, that is, receptors for which endogenous ligands are unknown. Commercially available screening formats to measure binding interactions of nuclear receptors to coregulatory proteins include fluorescence polarization assays, time-resolved fluorescence energy transfer (LANCE) and luminescent oxygen channeling (ALPHAScreen: Perkin Elmer, Wellesley, MA). These homogenous formats require tagging of both the nuclear receptor and the coregulatory protein with such methods as biotinylation or antibody linkage.

FP has been used to study the interaction of the Liver X Receptor (LXR) with a coactivator peptide from the endogenous coactivator protein, SRC-1 [22]. Recently identified using combinatorial phage display are higher affinity coactivator-like peptides for ER (dissociation constants in nM range versus μM range of native coregulators) containing the conserved LXXLL coactivator motif [29]. By using fluorescently-tagged coactivator-like peptides, FP has been used to monitor the ligand-induced recruitment or disruption of estrogen receptors alpha and beta (ERα and ERβ) binding to a fluorescently-tagged

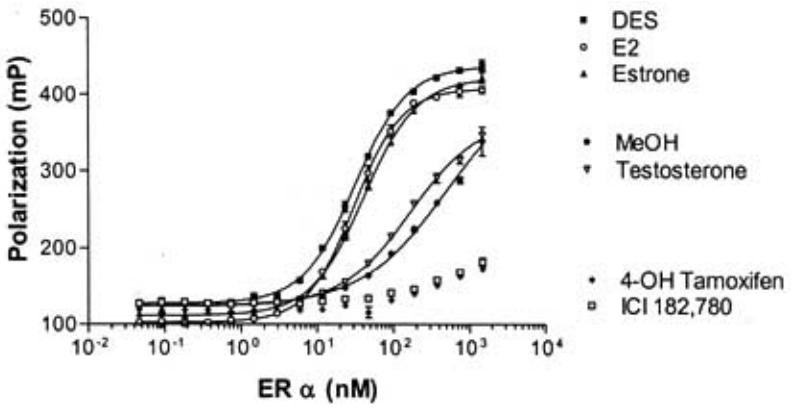

Fig. (4). ERα/coactivator binding.

ERα was serially diluted in a 384-well plate. Rhodamine-labeled coactivator peptide and ligand was added at a final concentration of 1 nM and 5 μM, respectively. Fluorescence polarization of each well was measured in the TECAN Ultra instrument (excitation 535 nm; emission 590 nm). Note that ERα shows greater affinity for the coactivator peptide in the presence of agonist ligands (DES, estradiol, estrone) than in the presence of non-ERα ligands (testosterone) or no ligand. Almost no binding could be detected in the presence of antagonist ligands (4-hydroxytamoxifen, ICI 182,780).

coactivator-like peptide. (Mary Ozers, PanVera, LLC, manuscript in preparation). Two formats have been applied to measure the strength of ER/coactivator binding. The equilibrium binding format uses increasing amounts of ER with saturating ligand [Fig. (**4**)], and in the dose-dependency binding format, ER and fluorescent coactivator are added to increasing concentrations of ligand [Fig. (**5**)].

These nuclear receptor/coactivator data show how FP can be used to measure protein conformation changes induced by small molecules. Specifically it shows in the same experiment that different small molecules can either increase or decrease affinity of the tracer, as shown with either an increase or decrease in polarization. As instrumentation improves with tighter measurements of FP, it will be easier to use larger protein domains in the binding experiments because even with a lower shift in polarization, it will be possible to develop an assay with an acceptable Z' factor of greater than 0.5 (personal communication CRI, Woburn, MA).

KINASES

In the past decade, drug discovery efforts have increasingly focused on targeting specific molecules in

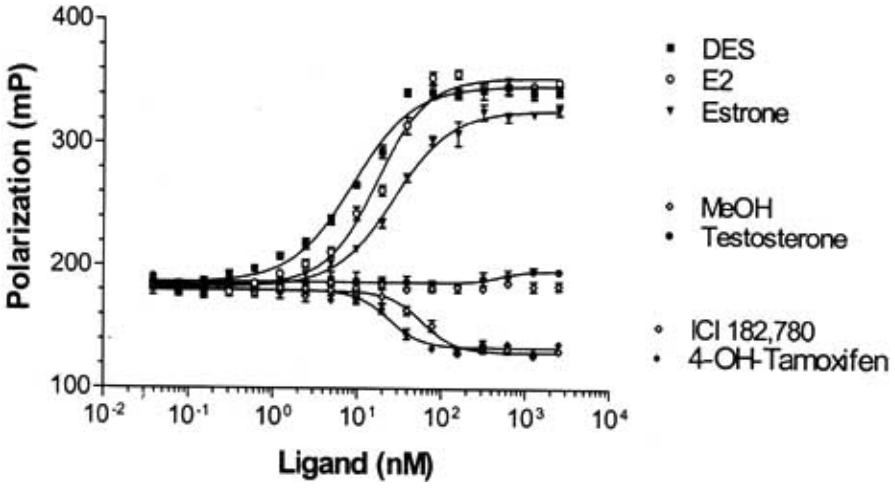

Fig. (5). ERα/coactivator binding.

Ligand was serially diluted in a 384-well plate. ERα and rhodamine-labeled coactivator peptide were added at a final concentration of 75 nM and 1 nM, respectively. Fluorescence polarization was measured in the TECAN Ultra instrument. Note that agonist ERα ligands promote the formation of ERα/coactivator complex, antagonists disrupt complex formation, and no ligand or non ligand has no effect.

disease-associated signaling pathways. Because of the number of kinase-dependent biochemical pathways that are implicated in human disease, many kinases are excellent targets for drug discovery [30-35]. There are approximately 500 kinase genes in the human genome and about 10-20% have been screened as drug targets. Many kinase inhibitors that bind in the ATP binding pocket are currently in clinical trials. Gleevec, a Bcr-Abl kinase inhibitor used to treat chronic myeloid leukemia, is the first to receive FDA approval [36]. Next to G-protein coupled receptors, kinases are the most commonly screened drug target class of proteins and kinase screening is still expanding [37].

Approximately 15% of all high-throughput screening using biochemical assays is done radioactively, [37] including kinase assays. In one example, kinase activity is measured via incorporation of radioactive [γ-^{32}P or γ-^{33}P] ATP into a peptide or protein substrate. The substrate is then bound to phosphocellulose (P-81) filter discs or precipitated with trichloroacetic acid (TCA). After several washing steps, the amount of radioactive product recovered is measured on a liquid scintillation counter [38,39]. There are drawbacks to these radioactive, non-homogeneous assays particularly in that they produce radioactive waste and can be challenging to automate. Several non-radioactive cocktail alternatives using FP have been developed for use in biochemical HTS assays [40-43].

By 2003, it is estimated that 10-14% of all high-throughput screening using biochemical assays, including kinase assays, will be performed using fluorescence polarization [37]. FP measurement in kinase assays is very sensitive and can easily detect phosphorylated substrates in low nanomolar concentrations. It is scalable and can be used in 1536 well plates [14]. A few methods have been developed to utilize FP to detect phosphorylated substrates and they are described briefly below.

FLUORESCENCE POLARIZATION IMMUNOASSAYS (FPIA'S)

FPIA's are competitive immunoassays that can use a phosphospecific antibody to distinguish a phosphorylated from a non-phosphorylated kinase substrate. They are used commercially to screen compound libraries for small molecules that modulate kinase activity and for more in depth analyses of enzyme kinetics, substrate specificity, and inhibitor characterization. The principle of this assay is based on the competitive binding between 1) a fluorescent phosphopeptide (tracer), 2) phosphopeptides or phosphoproteins generated during a kinase reaction, and 3) a phosphospecific antibody. The tracer binds to the antibody, resulting in a complex with a high polarization value. When this complex is added to a kinase reaction, the phosphorylated products bind to the antibody, liberating the tracer and causing the polarization value to decrease. The decrease in polarization signal is proportional to kinase activity, and small molecule modulation of kinase activity can be easily measured in either a kinetic or an endpoint assay. There is a very good correlation between an FP-based kinase assay and the amount of phosphate incorporated into the substrate (Fig. (**6**)).

There are several important issues in developing FPIA's for high-throughput screening and compound profiling in drug development. An assay must have a very pure fluorescent phosphopeptide tracer and a corresponding

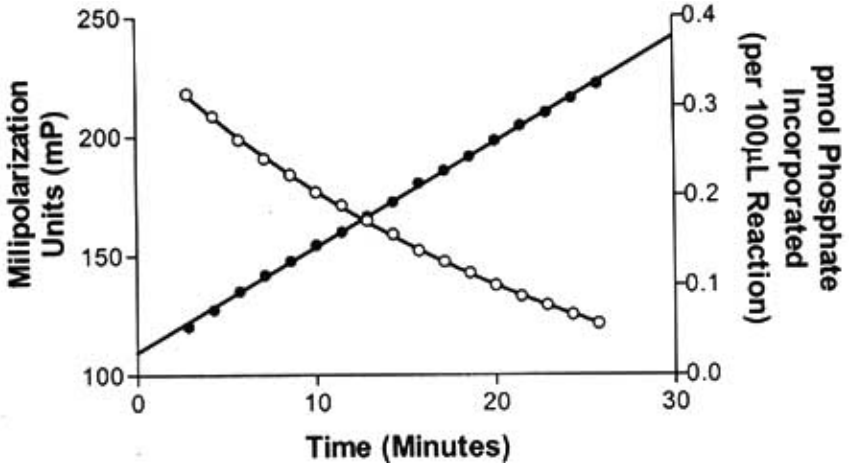

Fig. (6). The Relationship Between Polarization and the Incorporation of Phosphate Into Substrate.

The Epidermal Growth Factor Receptor (EGFR) kinase (0.5 nM, 0.13 U/100 µL) was pre-incubated in reaction buffer and 2.5 mM ATP followed by the addition of poly(Glu, Tyr) 4:1 copolymer substrate to 15 nM. The decrease in polarization was monitored as the reaction progressed (o) and the data were described by a single exponential decay with $mP_{highest}$ - mP_{lowest} = (183 ± 1) mP, k = (0.0135 ± 0.0005) min^{-1}, and mP_{lowest} = (38 ± 0.4) mP. The increase in the amount of phosphate incorporated (●) was calculated from these data using kinetic parameters from a standard curve (data not shown). The amount of phosphate transferred to substrate was plotted with respect to time. These data were then fitted using the equation for a straight line, with slope = initial velocity = (0.0117 ± 0.0001) pmol phosphate incorporated/min and intercept = (0.026 ± 0.001) pmol phosphate incorporated.

antibody with a tight affinity (low nM K_d) for the tracer and little or no affinity (uM K_d) for the unmodified substrate. The antibody/tracer complex must also have a relatively high shift in polarization compared to the tracer alone (150-300 mP preferred). However, as FP instrumentation improves, this high shift in polarization becomes less important in developing assays amenable to high throughput screening. FP kinase assays can have very high Z' factors [15], which depend on both the biochemistry as well as the plate reader precision.

With the FP platform, reactions may be conducted by measuring polarization in real-time as the reaction proceeds [Fig. (**7**)] or as an endpoint assay. The real-time assay is effective when equilibration among the phosphorylated product, antibody and tracer is rapid compared to the rate of phosphorylation. If this is not the case, the rate of phosphorylation can be underestimated by this method. To circumvent this issue, reaction aliquots can simply be quenched at desired time points with EDTA and incubated with antibody and tracer until equilibrium is reached before polarization is measured.

FP KINASE ASSAYS NOT REQUIRING ANTIBODIES

In the following two examples, molecules that can distinguish phosphorylated from non-phosphorylated peptides have been substituted for the antibody in the FPIA. The system from Molecular Devices (Sunnyvale, CA) relies on the specific binding of metal (M^{III}) coordination complexes to phosphate groups. In a microwell assay format, fluorescently labeled peptides are phosphorylated in a kinase reaction, and then the nanoparticles derivatized with metal (M^{III}) coordination complexes are added to the assay to bind the phosphorylated peptides but not the nonphosphorylated peptides. Binding causes an increase in the observed fluorescence polarization. It has an advantage in that the detection element of the system is not dependent on specific substrate peptide sequences. However, it has limitations in that the substrate sequence must not contain an excess of acidic residues and at least 5% of the substrate should be phosphorylated to get a reproducible signal. High ATP concentrations can also bind to the detection molecules and therefore either must be kept at low concentrations in the kinase reaction or be diluted prior to addition of the complex (Sportman, R. this volume).

POLYARGININE AND THIOPHOSPHORYLATION-BASED FP ASSAYS

Another variation of FP-based kinase assays takes advantage of the change in net ionic charge on a peptide substrate after it has been phosphorylated by a kinase [44]. In this technique, cationic polyamino acids (commonly polyarginine) are added to kinase reactions containing a fluorescently-labeled peptide substrate with a neutral (or near neutral) net charge. As the kinase reaction progresses, [30-35] the substrate becomes phosphorylated and the net ionic charge of the peptide will be lowered by the addition of the negatively charged phosphate. When the peptide's net ionic charge becomes negative, the polyarginine will selectively bind to the fluorescently labeled phosphopeptides, thus generating a measurable increase in polarization. Variations of this technique, called "Fluorescence Polarization in the presence of Polyarginine", can also be employed to measure phosphatases and proteases [45].

A kinase assay that uses ATPγS instead of ATP in the phosphorylation reaction has also been developed. This

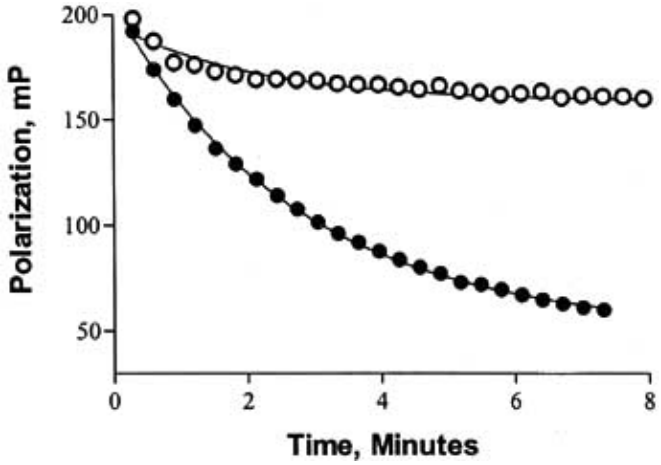

Fig. (7). The Phosphorylation of the Poly (Glu, Tyr) 4:1 Copolymer Catalyzed by Lyn A, Monitored in Real-Time

Lyn A (4.1 nM, 23 ng/100 µl), a Src enzyme of ≈ 56 kDa (Hibbs, M.L. *et al.* (1997) *Int. J. Biochem. Cell. Biol.* **29**:397-400) was incubated with 10 µM ATP in reaction buffer (20 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 2 mM $MnCl_2$, 0.05 mM Na_3VO_4, and 1 mM DTT) at 30 °C in the presence of substrate, 60 nM (2 ng/ul) poly (Glu, Tyr) 4:1 copolymer. Polarization was monitored in real-time in the Beacon 2000 (490 nM excitation, 525 nM emission), dropping from 200 mP to 60 mP in about 7 minutes (•). However, under identical conditions in the presence of 20 µM PP1 (a Src inhibitor, Hanke, J.H. *et al.* (1996) *J. Biol. Chem.* **271**:695-701), polarization only dropped to about 162 mP in 7 minutes (o).

substitution results in the transfer of a thiophosphate group onto a fluorescently labeled acceptor peptide substrate. The reaction mixture is treated with a sulfur-reactive iodoacetyl derivative of biotin, which leads to a modification of the nucleophilic sulfur of the thiophosphate group and the generation of a fluorescently labeled, biotinylated molecule. Streptavidin is then added to the reaction to bind to all of the biotinylated molecules present, thus increasing the molecular volume of the thiophosphorylated and biotinylated kinase substrate [46]. The change in the substrate molecule's volume can then be detected by an increase in polarization. Although seemingly more labor/reagent intensive than other FP-kinase assays, the technology may be quite useful with robotic systems or as a secondary screen option.

G-PROTEIN COUPLED RECEPTORS

The G-Protein Coupled Receptors (GPCRs) represents a large and functionally diverse superfamily of proteins involved in a broad array of signaling pathways. These proteins represent the most highly screened [37] and important class of therapeutic targets with hundreds of pharmaceuticals developed to date. GPCRs are 7-transmembrane proteins which contain extracellular receptors that are responsive to a variety of stimuli including hormones, neurotransmitters, retinals, and peptides. When the surface receptor binds to an agonist ligand, a conformational change is induced which allows the receptor to interact with the heterotrimeric G-protein within the cell membrane. The receptor-G protein interaction catalyzes a guanine nucleotide exchange on the α subunit of the G protein from GDP to GTP, which leads to dissociation of the α and $\beta\gamma$ forms of the G protein complex. The activated GTP-bound form of the G protein is then able to signal effector proteins which regulate cellular components such as metabolic enzymes, ion channels, and transcriptional machinery [47-49].

Conventional methods for monitoring GPCR binding events require a filtration or separation step and require use of radioactivity. In an effort to increase the throughput and eliminate the need for radioactivity in GPCR drug discovery, several fluorescence-based homogenous assays have recently been developed [50]. These assay formats use technologies such as homogenous time-resolved fluorescence, fluorescent microvolume analysis technology, fluorescence correlation spectroscopy, and FP. The important distinction between FP and the other fluorescent assays is that FP measures all tracer signal, not just the tracer signal that is bound. Because of this, the key considerations in GPCR assay design: GPCR expression levels, ligand affinity, and fluorescent intensity of the ligand, are weighted differently in a FP-based GPCR assay [51]. Perhaps the most important factor is that the binding affinity of the fluorescent ligand be sufficiently high to allow for a polarization increase in the presence of relatively low concentration of receptor. In many instances, one can increase FP assay windows by increasing the receptor concentrations to levels well above the K_d of that receptor/ligand interaction. However, because GPCRs are typically present in the form of whole cells or membrane preparations, high receptor concentrations may not be possible. Also, light scatter and autofluorescence of cellular components may limit the practicality. Despite these factors, several FP-based high-throughput screening assays targeting GPCR binding have been developed at a reagent cost less than or equal to conventional radioisotope filtration assays. Z' values of 0.3 to 0.6 can be achieved despite a relatively small shift in polarization (50 to 90 mP) [50].

In addition to monitoring GPCR receptor binding, FP-based assays can be used to measure downstream signaling vents of agonist binding. For example, a functional FP assay using the corticotropin releasing factor subtype 2^{α} ($CRF2\alpha$) as a model system for the measuring cAMP production has been developed [52]. Within a single well on a microtiter plate, cells can be stimulated, lysed, and assayed for cAMP content. Quantitation of stimulated cAMP is achieved by its competition for a fluorescein analog of cAMP for a limited amount of antibody in a format similar to the FP immunoassay (FPIA) for kinases described earlier. Both agonist assays (stimulation of cAMP) and antagonist assays (inhibition of agonist-mediated cAMP stimulation) have been built.

CONCLUSION

In the past few years, fluorescence polarization has become an established method in drug discovery for both analytical and high-throughput applications. FP assays have been used primarily for screening but the assays can also be used in target validation and compound profiling. FP assays have been developed for nearly all the major target classes in drug discovery, including GPCR's, kinases, proteases, nuclear receptors, and phosphatases. The applications in drug discovery have advanced rapidly because both the biochemistry and the instrumentation have improved. Many complete primary screening assays are commercially available, including the protein targets. The first FP plate readers were pioneering but the newest instrument prototypes have laser excitation and such precise measurement that it enables pharma scientists to develop assays with very small shifts in polarization but a high degree of precision and reproducibility.

ABBREVIATIONS

DBD	=	DNA binding domain
DNA	=	Deoxyribonucleic acid
DTAF	=	Diaminotriazinylamino-fluorescein
ERE	=	Estrogen receptor/estrogen response element
FlAsH	=	Fluorescein arsenical hairpin
FP	=	Fluorescence Polarization
GR	=	Glucocorticoid receptor
IC_{50}	=	Inhibitory concentration 50%
LANCE	=	LANCE™ (Perkin-Elmer)
LBD	=	Ligand binding domain
LXR	=	Liver X Receptor

SAR = Structure-activity relationships

SPA = Scintillation proximity assays

ER = Estrogen receptor

TCA = Trichloroacetic acid

ATP = Adenosine triphosphate

HTS = High-throughput screening

FPIA = Fluorescence polarization immunoassays

EGFR = Epidermal growth factor receptor

EDTA = Ethylenediaminetetraacetic acid

GPCR = G-protein coupled receptor

GDP = Guanosine diphosphate

GTP = Guanosine triphosphate

cAMP = Cyclic adenosine monophosphate

PR = Progesterone Receptor

PRLBD = Progesterone receptor ligand binding domain

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