

CHAPTER 1

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INTRODUCTION

Fluorescence polarization (FP) is a powerful, but underutilized tool for studying molecular interactions by monitoring changes in the apparent size of fluorescently-labeled or inherently fluorescent molecules, often referred to as the tracer or ligand (Checovich *et al.*, 1995; Heyduk *et al.*, 1996; Jameson and Sawyer, 1995; Nasir and Jolley, 1999). It is unique among methods used to analyze molecular binding because it gives a direct, nearly instantaneous measure of a tracer's bound/free ratio.

FP enables the researcher to view molecular binding events in solution, allowing true equilibrium analysis into the low picomolar range (*i.e.*, with as little as 10 femtomoles/mL of sample stoichiometrically labeled with fluorescein). FP measurements do not affect samples, so they can be treated and reanalyzed in order to ascertain the effect on binding by such changes as pH, temperature, and salt concentration. In addition, because FP measurements are taken in "real-time," experiments are not limited to equilibrium binding studies. Kinetic analysis of association and dissociation reactions are routine with fluorescence polarization.

Because FP is a truly homogeneous technique, it does not require the separation of bound and free species. Methods that depend on separation are not only more time-consuming, but they disturb the reaction equilibrium and therefore prevent accurate quantification of binding. Alternative homogeneous fluorescent techniques, such as fluorescence resonance energy transfer (FRET) and homogeneous time-resolved fluorescence or HTRF® (Pope *et al.*, 1999) require multiple labeling reactions instead of one as in FP. Also, because the data generated from FRET and HTRF® is fluorescence intensity, there is a higher probability that artifacts from quenching and exogenous fluorescence will interfere with readings. FP is a ratiometric measurement and therefore less sensitive to these sources of interference.

Theory

First described by Perrin (1926), the theory of FP is based on the observation that when a small fluorescent molecule is excited with plane-polarized light, the emitted light is largely depolarized because molecules tumble rapidly in solution during its fluorescence lifetime (the time between excitation and emission). However, if the tracer is bound by a larger molecule its effective molecular volume is increased. The tracer's rotation is slowed so that the emitted light is in the same plane as the excitation energy. The bound and free states of the tracer each have an intrinsic polarization value: a high value for the bound state and a low value for the free state. The measured polarization is a weighted average of the two values, thus providing a direct measure of the fraction of tracer bound to receptor. An increase in molecular volume due to receptor-ligand (Bolger *et al.*, 1998), DNA-protein (Lundblad *et al.*, 1996; Ozers *et al.*, 1997), or peptide-protein binding (Wu *et al.*, 1997) or a decrease in molecular volume due to dissociation or enzymatic degradation (Bolger and Checovich, 1994; Bolger and Thompson, 1994) can be followed by FP.

- Bolger, R. and Checovich, W. (1994) *BioTechniques* **17**:585-9.
- Bolger, R. and Thompson, D. (1994) *Am. Biotechnol. Lab.* **12**:113-6.
- Bolger, R. *et al.* (1998) *Environ. Health Perspect.* **106**:551-7.
- Checovich, W. J. *et al.* (1995) *Nature* **375**:254-6.
- Heyduk, T. *et al.* (1996) *Meth. Enzymol.* **274**:492-503.
- Jameson, D.M. and Sawyer, W.H. (1995) *Meth. Enzymol.* **246**:283-300.
- Lundblad, J.R. *et al.* (1996) *Mol. Endocrinol.* **10**:607-12.
- Nasir, M.S. and Jolley, M.E. (1999) *Comb. Chem. High Throughput Screen.* **2**:177-90.
- Ozers, M.S. *et al.* (1997) *J. Biol. Chem.* **272**:30405-11.
- Perrin J. (1926) *Phys. Rad.* **1**:390-401.
- Pope, A.J. *et al.* (1999) *Drug Discovery Today* **4**:350-62.
- Wu, P. *et al.* (1997) *Anal. Biochem.* **249**:29-36.

As illustrated in **Equation 1.1**, the polarization value of a molecule is proportional to the molecule's rotational relaxation time, or the time it takes to rotate through an angle of 68.5°. Rotational relaxation time is related to viscosity (η), absolute temperature (T), molecular volume (V), and the gas constant (R).

$$\text{Equation 1.1: } \text{Polarization value} \propto \text{Rotational relaxation time} = \frac{3\eta V}{RT}$$

Therefore, if viscosity and temperature are held constant, polarization is directly related to the molecular volume (*i.e.*, molecular size). Changes in molecular volume can result from binding or dissociation of two or more molecules, degradation, or from conformational changes.

Fluorescence polarization detection is described schematically in **Figure 1-1**. Monochromatic light passes through a vertical polarizing filter and excites fluorescent molecules in the sample tube. Only those molecules that are oriented properly in the vertically polarized plane absorb light, become excited, and subsequently emit light. The emitted light is measured in both the horizontal and vertical planes.

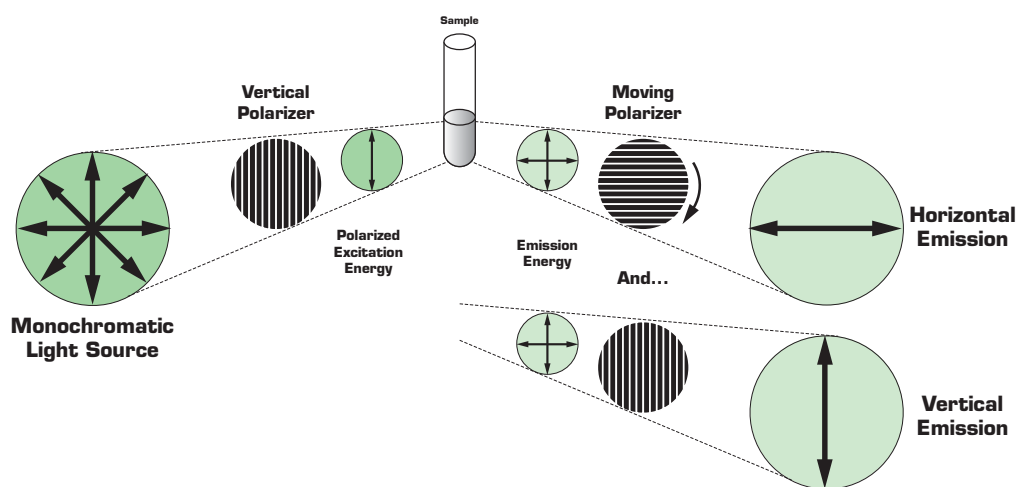


Figure 1-1. Schematic Representation of FP Detection. Monochromatic light passes through a vertical polarizing filter and excites fluorescent molecules in the sample tube. Only those molecules that are oriented properly in the vertically polarized plane absorb light, become excited, and subsequently emit light. The emitted light is measured in both the horizontal and vertical planes.

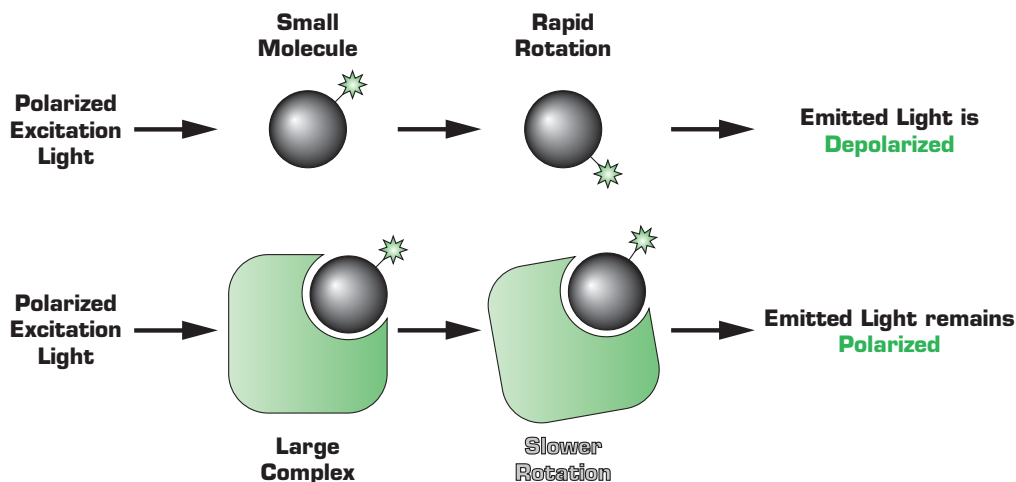
Polarization is calculated as shown in **Equation 1.2** and is a measure of the extent of molecular rotation during the period between excitation and emission.

$$\text{Equation 1.2: } \text{Polarization value (P)} = \frac{\text{Intensity}_{\text{vertical}} - \text{Intensity}_{\text{horizontal}}}{\text{Intensity}_{\text{vertical}} + \text{Intensity}_{\text{horizontal}}}$$

The polarization value, P , being a ratio of light intensities, is a dimensionless number, often expressed in millipolarization units (1 Polarization Unit = 1000 mP Units).

Illustrated examples of complexes with high and low polarization values are shown in **Figure 1-2**. Small molecules rotate quickly during the excited state, and upon emission, have low polarization values. Large molecules, in this case caused by the binding of a second molecule, rotate little during the excited state, and therefore have high polarization values.

Figure 1-2. Schematic of FP differences between small and large complexes. Small molecules rotate quickly during the excited state, and upon emission, have low polarization values. Large molecules, in this case caused by the binding of a second molecule, rotate little during the excited state, and therefore have high polarization values.



Anisotropy or Polarization?

The term “anisotropy” is sometimes used in literature associated with the FP field. Polarization and anisotropy are both derived from the measured vertical and horizontal intensities. The values are mathematically related and easily interconverted. Both values represent a weighted average of the bound versus unbound states of the fluorescent molecule.

While anisotropy and polarization share the same content of information, anisotropy values are mathematically easier to manipulate in many FP studies. A population of excited, identical molecules in solution will all have the same polarization value. If a portion of these molecules undergoes an apparent size change such that their rotational relaxation rate also changes, the observed polarization value represents an average of the component polarizations of all of the molecules. The polarization value of a mixture of molecules was described by Weber (1952) and is shown here as **Equation 1.3**:

$$\text{Equation 1.3: } \left(\frac{1}{P} - \frac{1}{3} \right)^{-1} = \sum_{i=1}^n f_i \left(\frac{1}{P_i} - \frac{1}{3} \right)^{-1}$$

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

$$\text{Equation 1.4: } A = \sum_{i=1}^n f_i \times A_i$$

Due to mathematical simplicity, anisotropy values are sometimes preferred because it is easier to deconvolute anisotropy values into their component values than it is with polarization values. It should be noted again that in the majority of applications, anisotropy does not give any additional information over polarization. The issue of using anisotropy versus polarization is discussed further in **Chapter 8**.

We generally use the term “Fluorescence Polarization” instead of “Fluorescence Anisotropy” because FP is most often the term used to describe the entire technology. In many applications that involve a minimum of curve analysis, we use polarization out of habit and tradition and because the error involved when using polarization instead of anisotropy is nominal.

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

THE BEACON® 2000 FLUORESCENCE POLARIZATION SYSTEM

Historically, fluorescence polarization had been used worldwide in the human diagnostic market for more than a decade (Jolley, 1981), but it had seen limited application in the broader research market due to the lack of sensitive, versatile, and affordable instrumentation. PanVera® developed one of the original instruments that met all of these requirements - the Beacon® 2000 Fluorescence Polarization System (PanVera® Part No. P2300). The Beacon® 2000 System, which is a single-tube instrument, continues to provide a rapid and reliable way to measure equilibrium binding or molecular degradation for a wide range of biological molecules including proteins, nucleic acids, carbohydrates, lipids, and drugs. The Beacon® 2000 Instrument was designed specifically to meet research needs. It is a compact, benchtop instrument that takes measurements on samples *directly in solution* and provides *picomolar* sensitivity (*i.e.*, low femtomoles/mL of sample stoichiometrically labeled with fluorescein). Applications as varied as protein-DNA interactions, immunoassays, protease assays, epitope mapping, DNA hybridization, and receptor-ligand binding studies are easily performed on the Beacon® 2000 System.

Because FP is now routinely used in the high-throughput screening environment, newer and faster multi-well plate-based instruments are often the tool of choice for these industrial applications.

HIGH-THROUGHPUT SCREENING USING FP

The tremendous challenge facing high-throughput screening (HTS) scientists today is to screen more compounds against more targets using more quantitative and robust methods without spending more money. Genomics efforts have flooded drug discovery with potential new drug targets (Drews, 1996). New parallel combinatorial synthesis methods are providing more compounds that must be screened for activity (Gallop *et al.*, 1994). The process for identifying new lead compounds must become more efficient. In order to increase the efficiency of HTS, new screening methods must be faster, cheaper, and more quantitative. Assays need to be miniaturized to decrease reagent costs and consumption of compound libraries. New assay formats must be homogeneous, requiring no separation of reaction components.

Fluorescent methods are rapidly becoming the primary detection format in HTS because they now approach the sensitivity of radioactive techniques and are amenable to homogeneous and miniaturized formats (Pope *et al.*, 1999). The increasing use of homogeneous fluorescence methods continues to be driven by a mandate within large pharmaceutical companies to significantly reduce the use of radioactivity in all facets of pharmaceutical research due to waste management issues. Homogeneous formats are desirable because these formats are “Mix-and-Read” without wash steps, multiple incubations, or separations required. Because FP measurements are made directly in solution, no perturbation of the sample is required, making the measurement faster and more quantitative than conventional methods.

- Drews, J. (1996) *Nat. Biotechnol.* **14**:1516-17.
- Gallop, M.A. *et al.* (1994) *J. Med. Chem.* **37**:1233-51.
- Jolley, M.E. (1981) *J. Anal. Toxicol.* **5**:236-40.
- Pope, A.J. *et al.* (1999) *Drug Discovery Today* **4**:350-62.

Since 1995, there have been a number of publications, listed in **Table 1-1** (adapted from Owicki, 2000), detailing the development of HTS assays using FP. As these articles demonstrate, FP has been most applicable when the HTS assay involves measuring changes in the fraction-bound of a small, fluorescently-labeled molecule and a large, unlabeled molecule. The kinase assays are actually FPIA's (FP immunoassay) where a phosphopeptide formed in a kinase reaction displaces a fluorescently-labeled phosphopeptide from a phospho-specific antibody. The nuclear receptor assays are ligand displacement assays where the affinity of test compounds for a receptor are measured by their ability to displace a bound fluorescent ligand. These and other assay types are described in detail later in this Guide.

Table 1-1. Publications detailing the development of HTS assays using FP.

Publication	Assay Type/Target Class
Deshpande <i>et al.</i> (1999)	Kinase
Parker <i>et al.</i> (2000)	Kinase
Wu <i>et al.</i> (2000)	Kinase
Parker <i>et al.</i> (2000)	Phosphatase
Parker <i>et al.</i> (2000)	Nuclear Receptors
Keating <i>et al.</i> (2000)	Protein-Protein
Lynch <i>et al.</i> (1997)	Protein-Protein
Lynch <i>et al.</i> (1999)	Protein-Protein
Wu <i>et al.</i> (1997)	Protein-Protein
Jolley (1996)	Protease
Levine <i>et al.</i> (1997)	Protease
Pope <i>et al.</i> (1999)	Protease
Chen <i>et al.</i> (1999)	Genomics
Pope <i>et al.</i> (1999)	Nucleic Acid
Allen <i>et al.</i> (2000)	GPCR
Banks <i>et al.</i> (2000)	GPCR
Pope <i>et al.</i> (1999)	Topoisomerase
Pope <i>et al.</i> (1999)	Cytokine Receptors
Li <i>et al.</i> (2000)	Transferase
Zhao <i>et al.</i> (1999)	Antimicrobials
Kauvar <i>et al.</i> (1995)	Protein-fluorescent probe binding
Sportsman <i>et al.</i> (1997)	Protein-fluorescent probe binding

- Allen, M. *et al.* (2000) *J. Biomol. Screen.* 5:63-70.
- Banks, P. *et al.* (2000) *J. Biomol. Screen.* 5:159-68.
- Chen, X. *et al.* (1999) *Genome Res.* 9:492-8.
- Deshpande, S. *et al.* (1999) *Prog. Biomed. Optics (SPIE)* 3603:261-61.
- Jolley, M. (1996) *J. Biomol. Screen.* 1:33-8.
- Kauvar, L.M. *et al.* (1995) *Chem. Biol.* 2:107-18.
- Keating, S. *et al.* (2000) *Prog. Biomed. Optics (SPIE)* 3913:128-137.
- Levine, L.M. *et al.* (1997) *Anal. Biochem.* 247:83-8.
- Li, Z. *et al.* (2000) *J. Biomol. Screen.* 5:31-7.
- Lynch, B.A. *et al.* (1997) *Anal. Biochem.* 247:77-82.
- Lynch, B. *et al.* (1999) *Anal. Biochem.* 275:62-73.
- Owicki, J.C. (2000) *J. Biomol. Screen.* 5:297-306.
- Parker, G.J. *et al.* (2000) *J. Biomol. Screen.* 5:77-88.
- Pope, A.J. *et al.* (1999) *Drug Discovery Today* 4:350-62.
- Sportsman, J.R. *et al.* (1997) *High Throughput Screening: the Discovery of Bioactive Substances*, pp. 389-400. Marcel Dekker, New York.
- Wu, P. *et al.* (1997) *Anal. Biochem.* 249:29-36.
- Wu, J.J. *et al.* (2000) *J. Biomol. Screen.* 5:23-30.
- Zhao, G. *et al.* (1999) *Antimicrob. Agents Chemother.* 43:1124-8.

The purpose of this Guide is to give the reader an intuitive feel for the utility of fluorescence polarization. HTS scientists will continue to increase their use of FP due to its unique combination of simplicity, speed, and robustness. Additional improvements in multiwell instrumentation, and the discovery of longer lifetime and longer wavelength fluorophores will expand its use even further.

Limitations of FP

FP requires relatively large changes in molecular volume for maximum signal (change in mP value). The FP value increases with molecular weight, but reaches a plateau level dependent on the fluorescence lifetime of the fluorophore. For a discussion of the impact of lifetime on FP values, see Pope *et al.*, (1999). For example, the lifetime of fluorescein is 4 nanoseconds, which is about the same amount of time required for a small molecule (<10 kDa) to randomize its orientation relative to the incident plane of excitation energy. The resulting fluorescence will therefore be depolarized. Binding of this molecule to a larger one will slow down the tumbling of the complex and the fluorescence will remain polarized. A lifetime of 4 nanoseconds is not optimum for observing the binding of a large protein (>30 kDa) to other proteins because the fluorescence is already highly polarized.

Being a ratiometric technique, FP is resistant to absorbance or color quenching from library compounds. However, fluorescence from these library compounds can cause artifacts. This can be dealt with directly by pre-reading the fluorescence in a well before addition of the fluorescent reagent. The background fluorescence can then be subtracted out before the FP value is calculated. Performing background subtraction on individual wells is often not possible during a primary screen, but usually performed in follow up screening on “flagged” compounds that demonstrated significant changes in fluorescence intensity. The probability of finding background fluorescence problems is reduced at higher wavelengths. Therefore using red-shifted probes in place of fluorescein will minimize background fluorescence interference.

Table 1-2 (adapted from Pope *et al.*, 1999) provides a brief listing of advantages and disadvantages of the most prominent homogeneous, fluorescence detection methods.

Table 1-2. Advantages and disadvantages of fluorescent formats for homogeneous high throughput screening assays		
	Advantages	Disadvantages
Fluorescence Intensity (FLINT)	<ul style="list-style-type: none"> • Simple • Suitable for fluorogenic assays • Readily miniaturized 	<ul style="list-style-type: none"> • No information for quality control • Sensitive to inner-filter and autofluorescence interference
Fluorescence Polarization (FP)	<ul style="list-style-type: none"> • simple, predictive • insensitive to inner-filter effects • ratiometric technique • suitable for small (<10 kDa) ligands 	<ul style="list-style-type: none"> • local motion effects • suitability limited by lifetime of dye, ligand size, and molecular weight change • can suffer from autofluorescence
Fluorescence Resonance Energy Transfer (FRET)	<ul style="list-style-type: none"> • suitable for short inter/intramolecular distances (<5 nm) • range of available donors and acceptors 	<ul style="list-style-type: none"> • requires multiple labels • sensitivity to inner-filter and autofluorescence interference • limited to short distances to obtain high signal changes
Time-Resolved energy Transfer (TRET)	<ul style="list-style-type: none"> • robust • suitable for long distances (5–10 nm) • reduced autofluorescence interference 	<ul style="list-style-type: none"> • requires multiple complex labels • limited choice of donors/acceptors

• Pope, A.J. *et al.* (1999) *Drug Discovery Today* 4:350-62.