

Fluorescence Polarization: An Analytical Tool for Immunoassay and Drug Discovery

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Abstract: Fluorescence polarization (FP) is an intrinsically powerful technique for the rapid and homogeneous analysis of molecular interactions in biological/chemical systems. The technique has been successfully used to diagnose various viral and infectious diseases in humans and animals, to monitor therapeutic drug levels and substances of abuse in body fluids and to determine food born pathogens, grain mycotoxins and pesticides. It has also been used in monitoring enzyme catalyzed hydrolysis, protein-protein interactions, DNA diagnostics and high throughput screening during the course of drug discovery. Work by various groups, including our own, have demonstrated that the technique can replace a substantial number of solid phase assays. FP, defined by the equation $P = [I_V - I_H] / [I_V + I_H]$ (where V and H are the vertical and horizontal components of the intensity I of emitted light respectively when excited by vertically plane polarized light), is independent of the intensity of the light and the concentration of the fluorophore. Hence it is functional in colored and cloudy solutions. The FP of a fluorophore is proportional to its rotational relaxation time, which in turn depends upon its molecular volume (or molecular weight) at constant temperature and solution viscosity. When a fluorophore-labeled ligand binds to a larger molecule, equilibrium is established rapidly and the FP increases. This property has been successfully exploited in many fields as described in this review.

Introduction

Fluorescence polarization (FP), first described by Perrin [1], is a powerful technique for the homogeneous analysis of molecular interactions. Other homogeneous fluorescence techniques include energy transfer, quenching and enhancement [2-4]. FP however, differs from other fluorescence techniques in that the polarization is proportional to the molecular size and does not depend on intensity changes. The sensitivity of the technique and the possibility of obtaining results rapidly without any separation and purification steps make FP more attractive than methods where one needs to physically separate the bound and unbound species before analysis. These characteristics of FP can bring two separate diagnostic groups, molecular diagnostics and immunodiagnostics together under one umbrella [5]. Since the introduction of FP principles in the beginning of this century by Perrin [1], the technique has gone through various cycles in its applications. Weber

developed the first instrument to measure binding isotherms in biomolecular interactions by FP in early 1950s [6-10]. After the work of Laurence [11] in the study of the binding of small molecules with proteins, Dandliker utilized the technique extensively in biological systems for the study of antigen-antibody and hormone-receptor interactions [12-15]. The technique was developed and utilized commercially to monitor therapeutic drug levels and to detect substances of abuse in human body fluids by Jolley et al. in the early 1980's at Abbott Laboratories [16-18]. The technique has recently been introduced for high throughput screening in the drug discovery process [19-22,36]. This was mainly due to the availability of automated FP instruments (Jolley Consulting and Research Inc.). Lately new instruments have been developed and are commercially available with varying degrees of throughput, sensitivity and automation [5,19-22]. With this advance in instrumentation, FP assays have come to the forefront in the last 4-5 years. Although the principles and theory of FP have been explained adequately in various papers and review articles [1-2, 6-8], a brief description is given below in order that the reader may clearly understand this technique.

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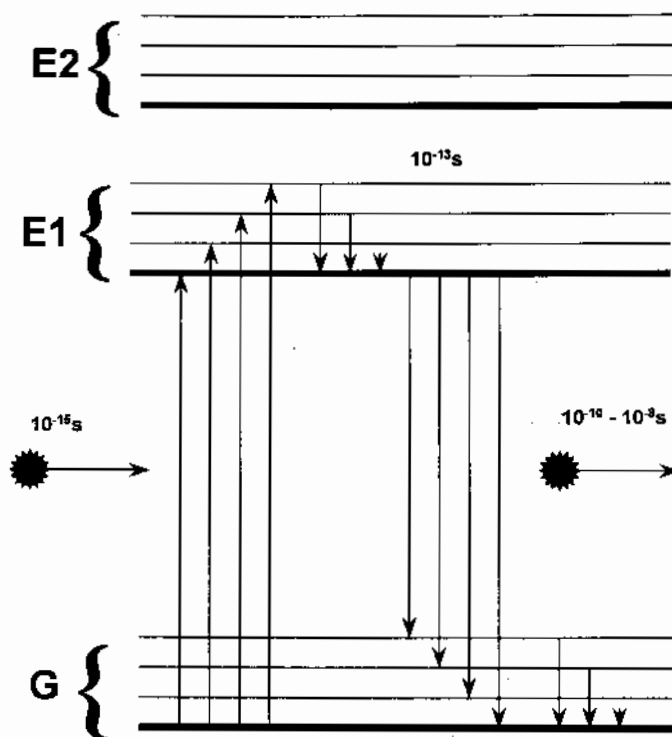


Fig. (1). Mechanism of fluorescence spectroscopy.

Theory

The principles of FP depend upon the excitation of a fluorescent molecule with plane polarized light. This results in the emission of photons in the planes parallel and perpendicular to the excitation plane and confers information about the local environment of the fluorophore [6,8]. Molecules in ground state **G** occupy the lowest vibrational energy level [Fig. (1)]. A source of photons of suitable energy can excite these molecules to the first singlet excited state **E₁** in a short time ($\sim$ fsec.). After this initial jump, the molecule loses excess vibrational energy and relaxes into the lowest vibrational energy level of **E₁**. After a period of time, τ (the fluorescence lifetime; defined as the time required for the fluorescence intensity to decay to $1/e$ of its zero-time intensity), the molecule emits a photon and eventually falls to the lowest vibrational energy level of **G**. This process takes place in the nsec. time frame. In most molecules, this process is lacking (excess energy is lost as heat or other intermolecular or intramolecular processes) and therefore only a few molecules are good fluorophores.

The fluorescence polarization of a fluorescent molecule is defined as

$$P = [I_V - I_H] / [I_V + I_H] \quad (1)$$

where I_V and I_H are the intensities of the vertical and horizontal components of the emitted light respectively

when the fluorescent molecule is excited by vertically plane polarized light. From this equation, it is clear that P is a dimensionless entity which does not depend upon the concentration and intensity of the fluorophore.

In Fig. (2) a fixed fluorescent molecule is represented by a line with two arrows. This is the excitation dipole of the fluorophore, the direction in which the molecule prefers to absorb light. For the sake of simplicity we assume that the excitation and emission dipoles are parallel (for some fluorophores these directions can change depending upon the structure of the molecule in its ground and excited states). By shining vertically plane polarized light on this vertically fixed molecule, the observed polarization value is 1 (100% polarization; 1000 mP; where 1 mP is defined as $P/1000$).

If a random array of fixed molecules [Fig. (3)] is illuminated by vertically plane polarized light, the molecules having excitation dipoles perpendicular to the plane of polarized light will not absorb and therefore not emit light. The molecules with other orientations will absorb and emit light to various degrees depending upon their alignment to the vertically polarized light. The emitted light will have a horizontal component and the system will have a limiting polarization (P_0) of 500 mP. Any polarization value greater than P_0 means that either the instrument is not calibrated properly or there is scattered light in the system.

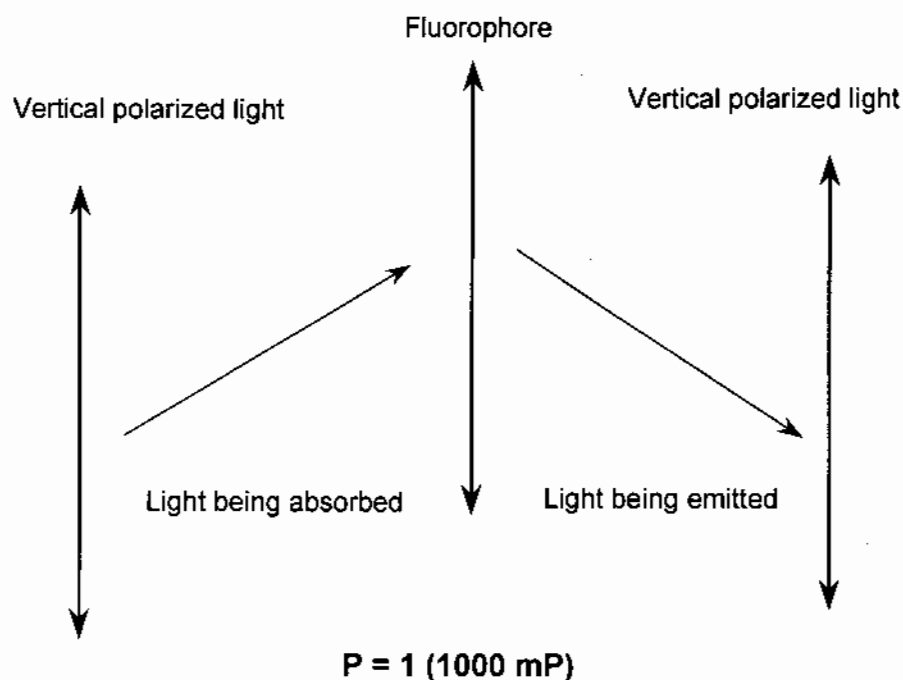


Fig. (2). Fluorescence polarization of a fixed fluorophore represented by a double headed arrow in vertically polarized light. Excitation and emission dipoles are parallel.

In a system where a random array of molecules are freely rotating [Fig. (4)], the polarization value will be between 0 and 500 mP depending upon the rotation rate of the molecules during the fluorescence lifetime of the excited state. If a molecule is small it will rotate faster and hence will have a smaller polarization value. Conversely, the larger molecules will have bigger polarization values due to their slow rotation.

At constant fluorescence lifetime the observed polarization is dependent on the rotational relaxation

time, ρ , for the molecule (the time required to rotate through an angle of θ , where $\cosine \theta = 1/e$ or $\sim 68.5^\circ$), which in turn depends upon the size of the molecule (molecular volume, V), temperature (T) and viscosity (η) of the system according to Stokes equation,

$$\rho = 3\eta V/RT \quad (2)$$

where R is the gas constant. The molecular volume is related to the molecular weight by the equation

$$V = vM \quad (3)$$

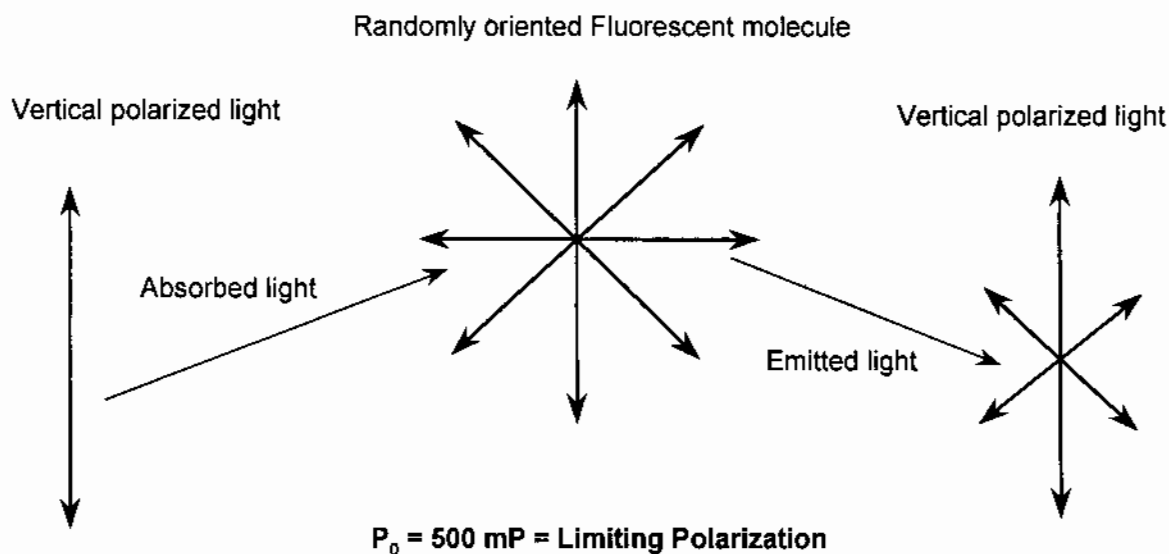


Fig. (3). Fluorescence polarization in a fixed system of randomly oriented molecules.

where v is the partial specific volume (ml/g) and M is the molecular weight of the molecule. Thus the higher the molecular weight of a molecule, the higher the rotational relaxation time will be (~4.3 nsec. for fluorescein and ~100 nsec. for immunoglobulins).

Perrin [1] defined the relationship between fluorescence polarization, fluorescence lifetime and rotational relaxation time in his pioneer work as follows:

$$(1/P - 1/3) = (1/P_0 - 1/3)(1 + 3\tau/\rho) \quad (4)$$

Combination of equations 2 and 4, and substituting M for V gives

$$1/P = 1/P_0 + \{1/P_0 - 1/3\} \times RT/vM \times \tau/\eta \quad (5)$$

It is clear from equation 5 that at infinitely high molecular weight and viscosity, and infinitely short lifetime, the limiting polarization will be the same as observed polarization. This suggests the limitation of FP. As the size of the molecule increases the difference between observed and limiting polarization decreases and therefore the sensitivity decreases accordingly.

In conclusion, the observed polarization value gives a direct measure of the molecular size of the analyte at constant temperature and viscosity. Since FP is a fundamental property of the molecule, a change in polarization value in a reaction mixture is directly used to monitor the change in molecular size in the process of assay development.

diseases and drug discovery processes. Because the reaction mixtures are analyzed in solution without separation the technique is perfectly suitable for kinetic and equilibrium studies. The central point is the development of a suitable fluorescent tracer which gives a maximum polarization change and minimum "propeller effect". The latter is a term used to describe the phenomenon whereby, although binding has occurred, little polarization shift is observed. This is caused by the uncoupling of the fluorophore and binding site due to a long, flexible linkage. To attain the best results one must employ the shortest and most rigid linkage possible between the fluorophore and the ligand. In addition one must achieve a high quantum yield and appropriate fluorescence lifetime. Traditionally FP has been employed for the study of small molecule-large molecule interactions but larger proteins (MW ~ 60 KDa) have been studied successfully [88-91,93,94]. The technique has been successfully employed in many diagnostic fields including the detection and quantitation of antigens or antibodies based upon antibody-antigen interactions, protein-DNA interactions, protein-RNA interactions, DNA detection and the monitoring of enzymatic reactions.

For ligand-receptor binding in this technique, the tracer purity and the position of labeling are very important. In order to make full use of FP one should be familiar with the Law of Mass Action. When a ligand (L) binds reversibly to a receptor (R) to form a complex (LR)



At equilibrium, the rate of forward reaction is equal to the rate of backward reaction. Therefore;

$$k_1[L][R] = k_2[LR] \quad (6)$$

Applications

Fluorescence polarization is used for the analysis of various species helpful in the diagnosis of various

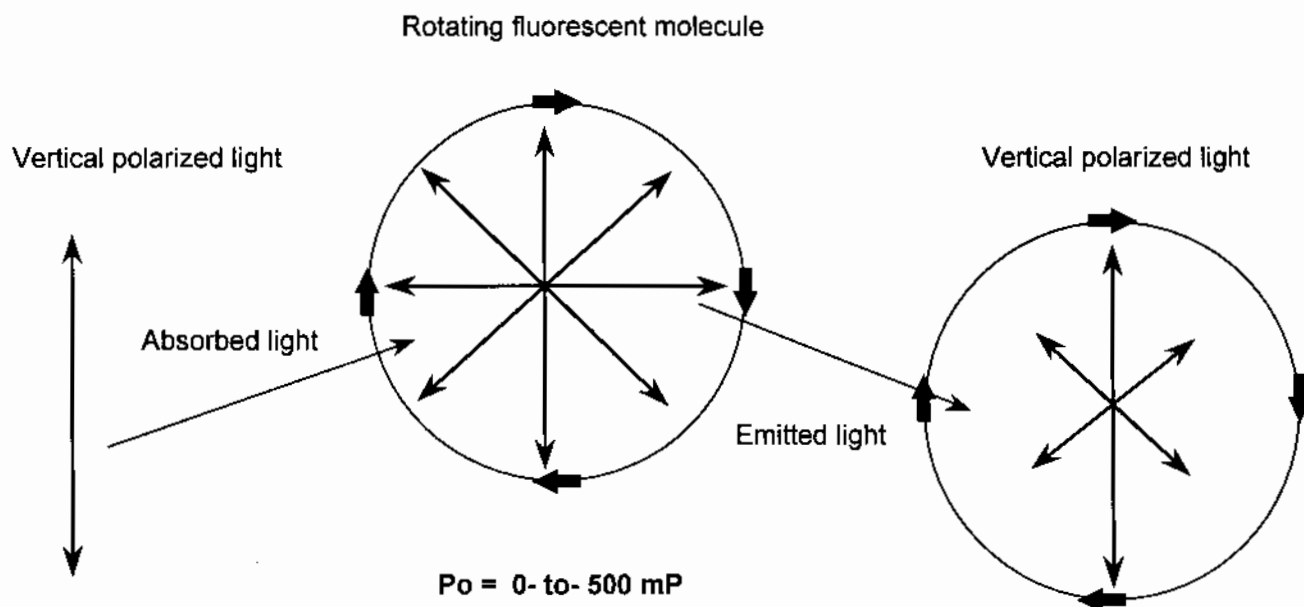


Fig. (4). Fluorescence Polarization in a real system of a random array of freely rotating molecules.

where [L], [R] and [LR] are the concentrations of L, R and LR respectively and k_1 and k_2 are the association and dissociation rate constants respectively. The association constant (K_a) and dissociation constant (K_d) are defined as:

$$K_a = 1/K_d = k_1/k_2 = [LR]/[L][R] \quad (7)$$

At 1/2 tracer binding (in this case the ligand) to the receptor

$[L] = [LR]$ and therefore

$$K_d = [R]$$

irrespective of the ligand concentration. Since

$$[Rt] = [Rb] + [R] = [Lt]/2 + K_d \quad (8)$$

$$[K_d] = [Rt] - [Lt]/2 \quad (9)$$

where [Rt], [Rb], and [Lt] are the concentrations of total receptor, bound receptor and total ligand respectively. Therefore with weak to moderate affinity systems ($K_d > 1\text{nM}$) a good estimate of the K_d for the tracer-receptor interaction may be obtained merely by titrating the tracer with receptor and calculating [Rt] at half tracer binding, ignoring [Rb]. For definitive determination of the K_d other, more sophisticated methods must be used [95].

Furthermore, the higher the binding constant, the lower the amount of receptor is needed for an assay and vice versa. For 10% tracer binding

$$[L] = [LR]/9 \quad (10)$$

and from equation (7)

$$[R] = K_d/9$$

$$[Rb] = [Lt]/10$$

and

$$[Rt] = K_d/9 + [Lt]/10 \quad (11)$$

Similarly for 90% tracer binding

$$[Rt] = 9K_d + 9[Lt]/10. \quad (12)$$

This means that to go from 10% tracer binding to 90% tracer binding, for moderate and low affinity systems where we can ignore Lt, one needs 81 times the amount of receptor. It is therefore very important to have an assay with as good a shift in polarization as possible in order to conserve receptor.

The affinities of drugs and drug candidates for their receptors is a very important consideration in drug

discovery. This can be derived from knowledge of the K_d of the tracer and the drug's IC_{50} (the concentration of the drug when tracer binding is half inhibited) using the Cheng-Prusoff correction [95] as follows:

$$IC_{50} = K_{di} (1 + [T]/K_{dt}) \quad (13)$$

where K_{di} is the K_d for the inhibitor, K_{dt} is the K_d for the tracer and [T] is total tracer concentration. Since this equation is derived from enzyme kinetics, instead of determining the true K_{di} one gets an approximate value, although an exact correction has been published [96]. IC_{50} is used to estimate the relative affinities of a number of inhibiting ligands. If the true affinity of one of them is known by another method, such as a radioreceptor assay or FP [97,98] then the affinities of the other inhibitors can be calculated.

A. Antibody-Antigen Interactions

These immunoassays were first utilized in the 1960s and have since been performed routinely by various groups [2,12-18,24-25]. An antigen or its derivative is labeled with a fluorescent molecule and the polarization is measured. Upon reacting with a specific antibody for the antigen, the polarization increases. Depending upon the quantity of unlabeled antigen in the sample, a competition occurs between the fluorescent-labeled and unlabeled antigen and the polarization decreases accordingly. Thus the observed polarization is correlated with the antigen concentration in the unknown sample [Fig. (5)] [12-13]. An optimum antibody-labeled antigen sample ratio is important for these studies to obtain the desired standard curve. The method has many advantages over ELISA and RIA. Because of the sequential washing and incubation steps in ELISA, equilibrium may be disturbed [26-28]. In ELISA compounds adsorbed to the solid phase may have different affinities than in solution. The adsorbed antigens may denature with time [29], and at the solid phase steric hindrance might occur for larger molecules [30]. Consequently the absence of washing steps, the reduced chances for contamination, and the absence of radioactive waste are advantages of FP.

1. Detection of Toxins in Grains and Environmental Samples

In view of the involvement of various mycotoxins in human, animal and grain diseases, rapid and cheaper field tests for the determination of these mycotoxins are becoming an urgent need. Typically HPLC and other chromatographic methods are used for mycotoxin analysis [31-33]. Aside from being labor intensive and expensive, these tests are time consuming and are not suitable for field testing. Usually the samples have to be submitted to reference

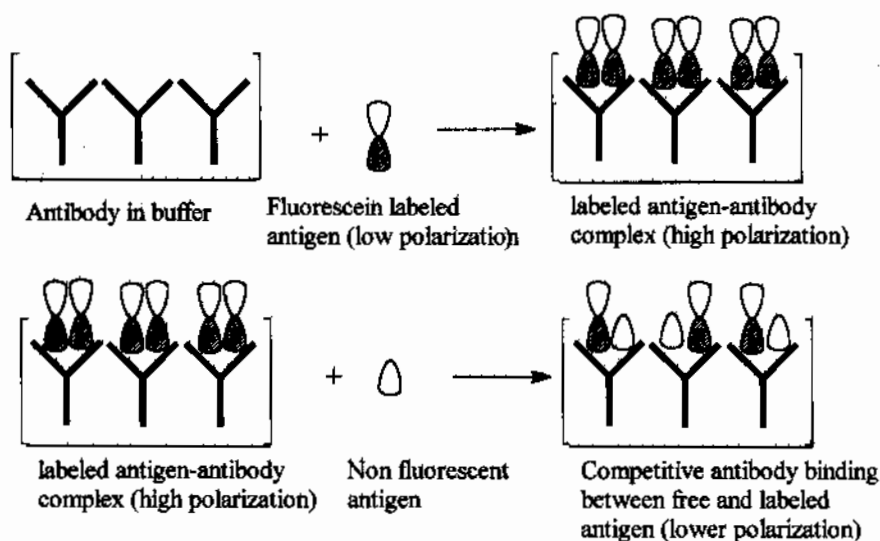


Fig. (5). Principle of small molecule detection based upon fluorophore labeled antigen-antibody interactions.

laboratories to be tested at later stages. FP, with its latest developments in simple and relatively inexpensive instrumentation, is a technique of great potential in this area of research. A mycotoxin antigen of interest is labeled with a suitable fluorescent molecule (tracer). Mycotoxin is extracted from grains with a suitable solvent and the sample is added into the antibody solution. Tracer is added and its fluorescence polarization is measured, which is inversely related to the amount of free mycotoxin. Dasgupta et. al. developed an assay for the detection of oleander poisoning in plants utilizing FP immunoassays for serum digitoxin and a polyclonal rabbit antibody [37]. Because of structural similarities of oleanderin with digitoxin the assay works nicely for oleandrin. They demonstrated that the assay is more sensitive than the FP assay of digoxin [38]. Rogers et. al. have developed a rapid (7-15 minutes), simple and, in essence, portable FP assay for the measurement of 2,4-dichlorophenoxyacetic acid (a common pesticide in environmental samples) using antibody or antigen-coated methyl methacrylate beads. The assay was demonstrated in buffer saline solutions as well as in aquatic environmental media [39]. In addition, Eremin et. al. have utilized FP extensively for rapid detection of various pesticides and herbicides [34-35].

2. Detection of Serum Proteins/ Antibodies

Serum protein/antibody detection in animals and humans is important in the diagnosis of various disease entities. Many methods, including RIA and ELISA, are used regularly for this purpose. But a rapid, cheap and specific method is still needed. FP assays have been developed for the detection of serum proteins in various disease states. Our group recently demonstrated the use of this technique for the rapid

detection of the presence of antibodies to *Mycobacterium bovis* in infected species (cow, elk, llama and bison) by using the labeled *Mycobacterium bovis* secretory protein (MPB70; M.Wt. ~ 18-23 KDa) [41]. With this method, infected animals could be diagnosed and separated from healthy animals. Serum samples from many animals infected with *M. bovis*, when combined with fluorophore-labeled protein after 50 fold dilution, resulted in an increase in polarization values. The samples from uninfected animals did not exhibit any increases in polarization and showed values similar to samples without any sera. The test is easier and simpler than ELISA and rapid (~10 minutes for a complete assay). Our group also developed an assay for the rapid determination of *Brucella abortus* antibody by labeling the O-polysaccharide prepared from *Brucella abortus* lipopolysaccharide with a molecular weight of ~20-30 KDa in infected cows [42]. The method is very sensitive, specific and is much faster (~2 to 3 minutes total test time) than all the available tests. Jiskoot, et al. measured the affinity constant of a monoclonal antibody-peptide complex and the average number of antigen-binding sites to antibody (MN₁₂, mouse IgG_{2a}) specific for *Meningococcal* strain protein [29]. They also demonstrated the use of this technique as an analytical tool in monoclonal antibody preparation.

3. Detection of Hormones

The importance of various hormones in the regulation of tissues and organs and their role in various diseases makes it important to find a sensitive and reliable diagnostic technique for the accurate, fast and reproducible determination of these hormones in serum, plasma and other body fluids. Hormone-binding interactions have been studied historically by radiolabeling. In FP, hormones of interest are labeled

with a fluorescein derivative. The system works like the antigen-antibody reaction described above. A hormone sensitive tissue or soluble receptor of proper concentration is added in a cuvette containing buffer and, after blanking, the fluorescent labeled hormone is added to get a stable polarization value. In a similar way the unlabeled hormone, containing sample, is mixed with buffer containing the hormone sensitive tissue or soluble receptor and, after blanking, the final polarization value is noted after the addition of fluorescein-labeled hormone. Levison et al. used this technique for hormone detection (estradiol, prolactin and growth hormone) in the early 1960s [15]. Kabayashi et al. developed an immunoassay for cortisol using fluorescence polarization in 1979 [43]. Al-Ansari et al., in 1983, developed a FP based immunoassay for cortisol determination in serum and saliva samples [46]. A significant improvement in FP instruments has simplified this process to a great extent. Choi et al. recently developed an immunoassay using the TDX (Abbott Labs.) to determine the levels of progesterone (a steroid hormone) in urine samples. The study describes the effect of immunogen and tracer conjugation sites on the sensitivity and specificity of the assay. Tracers were prepared by conjugating EDF (ethylenediamine fluorescein thiocarbonyl) to two different sites of progesterone similar to the immunogen preparation for antibody preparation [45]. Adamczyk, et al. have also described a FP immunoassay for the quantification of testosterone by using 1 α -(3'-carboxypropyl)-4-androsten-17 β -ol-3-one (a synthetic analog of testosterone) for immunogen and tracer preparation [44]. Wei et al. labeled a synthetic peptide (having sequence similar to the sequence located near the C-terminus of hCG β chain and with high affinity for Mab) with tetramethylrhodamine for the analysis of human chorionic gonadotrophin (hCG). Using FP, they detected hCG in nanomolar concentrations without any significant non-specific binding [40].

4. Src-SH₂ Binding Assays

The SH₂ domain is an important region within the tyrosine kinase Src family and has been implicated as an important therapeutic target in many diseases including cancer and osteoporosis. The development of various drug molecules as inhibitors of SH₂ interactions requires the ability of an assay to test libraries of compounds or natural product extracts as drug candidates. The assays must be reproducible and simple. Lynch, et al., described a high throughput screening assay for Src-SH₂ binding by synthesizing a carboxyfluorescein-coupled pentapeptide (based upon a known Src-SH₂ high affinity sequence) [63]. The assay is not affected even with ~ 20% DMSO as a solvent. This property makes the assay suitable for

compounds (inhibitors) usually insoluble in other solvents.

Wu, et al., recently developed a high throughput FP assay for the measurement of the interaction between an interferon- γ receptor derived phosphotyrosine-containing peptide and STAT (signal transducers and activators of transcription) using a 96 well automated FP instrument [19]. The assay gives binding constants similar to other methods but has obvious advantages as described earlier. Seethala et al. have recently developed an assay for tyrosine kinase by incubating a fluorescein-labeled peptide substrate with kinase and anti-phosphotyrosine antibody. During this incubation a phosphorylated peptide product is formed which reacts with the antibody and an increase in polarization is observed. The assay is comparable to the ³²PO₄ transfer assay and can detect inhibitors. This group has compared the FP assay with other techniques and has shown that the FP assay is more favorable compared to other methods [64-65].

5. Protein-DNA Interactions

The study of protein-DNA interactions using a sensitive and rapid technique is essential due to their involvement in the control of development and differentiation at various levels [3]. These interactions are traditionally measured using gel electrophoresis, which has many drawbacks including complex dissociation during electrophoresis [47]. With the advent of new developments in instrumentation, these interactions are easily and reliably studied using FP. A change in polarization values is monitored by titrating the protein into the fluorophore-labeled oligonucleotide solution [Fig. (6)]. Different research groups are using this technique for the determination of these interactions on a routine basis. LeTilley, et al., established a useful application of this technique by determining the dissociation constants of single and dimer trp repressor protein (TP), complexed with trp operator DNA, by labeling a 25-base oligonucleotide (containing trp operator sequence) with fluorescein [48]. Kwok et al. studied the interactions between a transcription factor (CREB) and a nuclear protein (CBP). The change in the interaction affinity between CBP and CREB upon phosphorylation of CREB was quantitated using a 23-based oligodeoxynucleotide labeled with fluorescein and titrating the CBP into phosphorylated and unphosphorylated CREB [49]. Hill et al. recently published an extensive study of equilibrium binding between various DNA binding proteins and labeled oligonucleotides. Using FP they monitored the equilibrium between fluorescein labeled DNA and the binding protein by titration with increasing amounts of protein [3].

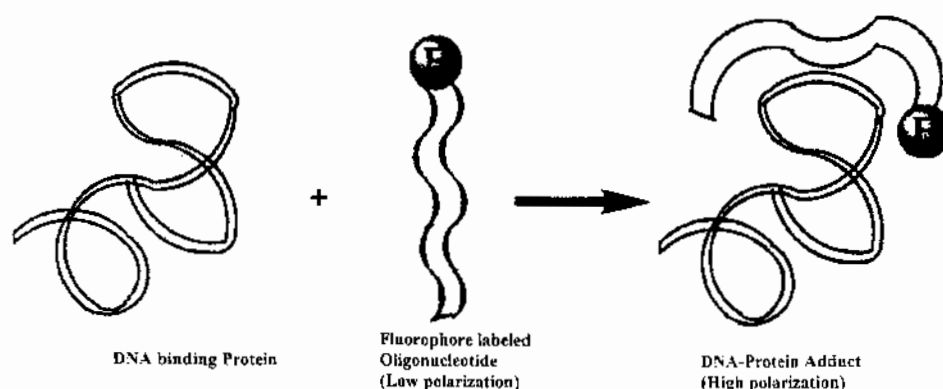


Fig. (6). Principle of polarization change during protein-DNA interactions.

6. Detection of Carbohydrates

Carbohydrates, including saccharides, polysaccharides, glycolipids and glycoproteins, are of enormous biological importance in various diseases and also as energy stores. Although a simple and robust FP assay for these compounds would be significant, FP has not yet been utilized for the analysis of this class of compounds. Nevertheless, using suitable fluorophore labels, FP could be utilized for carbohydrate determination. For example, in 1973 the Teichberg group studied changes in FP during saccharide-lysozyme interactions in hen white eggs. Using the polarization dependence of lysozyme and iodine-oxidized lysozyme on the saccharide concentration, they were able to imply the simultaneous binding of two saccharide molecules to the lysozyme cleft [50].

B. Enzymatic Reactions

The study of enzyme hydrolysis and their inhibition is an active area of drug discovery research. For example, various proteases are implicated in inflammatory disorders, infectious diseases, apoptosis and metastatic tumor progression [51-52]. Many protease assays in use are cumbersome, require separation, aliquot removal and the use of radioactive labeled

substrates. Fluorescent substrates have been utilized to solve these problems but lack sensitivity and specificity [53-54]. FP has been used for the study of proteases [21,55-60], DNases [61], RNases [62] and α -amylase [58] actions by following the degradation of fluorescein labeled substrates of high polarization. Upon degrading into smaller molecules, the fluorophores give lower polarization values. Thus a change in polarization of the fluorescent substrate upon protease action is monitored [Fig. (7)]. The method offers many advantages over other techniques.

Spencer et al. developed protease assays using fluorescein-labeled protein substrates in 1973 [23] and a detailed study of protease assay development using FP was carried out by Maeda in the late 1970s [55]. Proteolytic activities of various enzymes including trypsin, papain and pronase were measured using different enzyme concentrations and their protein substrates labeled with FITC. These substrates included fibrinogen, IgG and ribonuclease respectively. The results showed a near linear decrease in polarization with time due to the apparent decrease in molecular weight of the substrates with hydrolysis. He also demonstrated the proteolytic inhibition of papain or trypsin using various protease inhibitors (leupeptin, soybean trypsin inhibitor or antipain) [55]. A group at Abbott Laboratories employed fluorescein-labeled

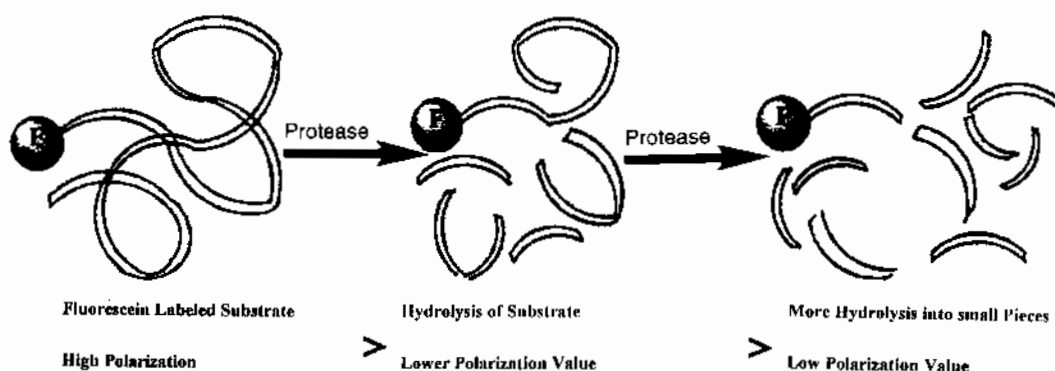


Fig. (7). Principle of fluorescence polarization measurements during enzyme hydrolysis.

amylose and other saccharide substrates for the rapid amylase determination in serum and urine utilizing fluorescence polarization [58]. Schade, et al. recently demonstrated the cell-associated or extracellular protease activity of bacteria without the isolation of bacteria. Using FITC- α -casein as substrate the protease activity was determined in 4 min on 10^7 *T. denticola* cells [59]. Due to the pH sensitivity of FITC, the method could not be utilized at low pH values. Later a new pH-independent fluorescent protein substrate (workable in the pH range of 2-11) was developed based upon BODIPY-FL- α -casein [21,60]. Protease assay kinetics were measured for pepsin, papain and proteinase K using this substrate in 1 to 5 min. Only 1-10 μ l of a growing culture was necessary to measure the protease activity of *Porphyromonas gingivalis* or *Treponema denticola*.

Levine et al., at Monsanto, designed a FP assay for proteolytic cleavage measurement of a peptide substrate specific for human cytomegalovirus protease in a 96 well format. The amino-terminus of the substrate was biotinylated and the fluorophore was put near the carboxy-terminus to have a protein-binding ligand and the fluorophore on the opposite sides of the peptide bond. Because of the specificity of the substrate for the proteases of interest, this protease assay is more useful than previously described assays and can be further utilized by designing specific substrates. The ability of this assay to study the inhibition activity of 3,4-dichloroisocoumarin (a cytomegalovirus protease inhibitor) suggested the versatility of this technique for the study of protease inhibitors [56].

C. Molecular Diagnosis

DNA detection and quantitation is important in a variety of biological applications including viral diagnosis and forensic analysis of DNA. There are many nucleic acid based infectious diseases detection methods, including DNA probes without amplification, detection of amplified products by a microtiter plate assay, gel electrophoresis, absorption measurements, fluorescence spectroscopy, dipstick assays and direct sequence analysis [66-70]. Most of these methods are heterogeneous, take considerable handling and time (4 to 6 hr), have poor sensitivity, encounter interference from other materials and contamination problems [71-72]. In many cases additional steps are needed to control these problems [73-74]. Microbial detection with high sensitivity and specificity has been facilitated with the advent of target amplification techniques including PCR [71,75-76], TMA [77], NASBA [78], LCR [72,79], SDA [80] and others [67]. However, a suitable detection system without the

above mentioned problems is still needed. FP as a DNA detection technique has recently been used and has many advantages over other methods. This could be a closed tube homogeneous technique without the need of separation of various reagents which prevents any kind of contamination problem. Probes can be labeled internally or at the 3' or 5' positions. In most of the cases the selection of fluorescein derivative and the length of the chain between fluorescein derivative and probe are very important. Common observations reveal that DTAF ((dichlorotriazinyl)aminofluorescein) derivatives of fluorescein are better than FITC (fluorescein isothiocyanate) derivatives. The chain length between probe and fluorescein molecule should be as small as possible. However, many times the steric hindrance can play its role and ultimately an experimentally compromised chain length has to be chosen. Karube used FP for the rapid determination of methicillin resistant *Staphylococcus aureus* DNA hybridization rate [81-82]. Using a suitable temperature and sodium chloride concentration detection of DNA hybridization was easily determined in ~10 minutes. Gibson, et al., [83] recently used FP to detect PCR-amplified DNA by combining the amplification refractory mutation system (ARMS) with FP in a 96-well plate format. Oligonucleotide probes were labeled with fluorescein dyes and mixed with the amplification matrix. The genotype analysis was in full agreement with conventional ARMS detection methods and did not have any contamination problems [83]. Walker, et al., have developed a new isothermal technique, SDA (strand displacement amplification), for DNA amplifications. SDA is an in vitro non-PCR technique that amplifies the target DNA exponentially (~ 10^{10} fold) in ~10-15 min through repeated nicking, strand displacement and priming of displaced strands [84-87]. It is based upon the ability of a restriction enzyme to nick a hemimodified recognition site and the ability of a polymerase to displace a downstream DNA strand during replication. With simultaneous amplification and detection the technique detects DNA rapidly and is very sensitive. A fluorophore attached to a single strand oligodeoxy-nucleotide possesses a low polarization. Hybridization causes the oligodeoxynucleotide to possess a higher polarization which can be monitored to detect very small amounts of DNA. Except for the initial denaturing step, it is an isothermal technique that enables detection of small amounts of DNA from different organisms and has advantages over other amplification techniques such as PCR and LCR [84-85]. The same group has recently developed a method for the simultaneous amplification and detection of *Chlamydia trachomatis* and *Mycobacterium tuberculosis* DNA using SDA in less than an hour [86-87].

1. Advantages of Fluorescence Polarization Over Other Techniques

FP has some obvious advantages over other techniques including other fluorescence techniques which deal with intensity change during a reaction. Because the horizontal and vertical components of intensity are internally controlled, the assays are immune to inner filter effects. The assays work very well in colored and cloudy solutions. These assays are very easy to develop (because of the very few steps in development) and do not depend upon wavelength or intensity changes. Unlike other fluorescence techniques there is no energy transfer requirement. The assays work with crude receptors obviating the need for receptor purification and labeling. There is no need of radioisotopes and enzymes (thus no disposal problems) and no solid phase artifacts. In some cases only a single incubation with a preformed tracer-receptor reagent may be performed and, since the technique does not require washing steps, the system is easily automatable. Because the system is relatively insensitive to changes in instrument and drift, only a little instrument standardization is needed. A ligand is labeled and titrated with the receptor. This is then titrated in the presence of an excess of unlabeled ligand to find any nonspecific binding. Finally a suitable tracer and tracer concentration is decided upon.

2. Limitations of Fluorescence Polarization

One of the main limitations of FP in assay development is that the difference between the size of analyte and receptor should be significant enough to give a reasonable polarization change. Since the excited state lifetime of fluorescein (a commonly used fluorophore) is ~4 ns FP is more suitable for relatively small antigens. However, the technology has successfully been used for analytes using tracers as large as 60 KDa. Lakowicz et al. have demonstrated the use of metal-ligand complexes with long fluorescence lifetimes as fluorophore labels for higher molecular weight antigens to overcome this limitation. They synthesized some laser diode-excitable metal-ligand fluorophores (Ru, Os) where they claim that by changing the metal, the decay time of the fluorophore can be varied radically (in the range of 19-300 nsec.) [88-90]. They have used these fluorophores for the detection of high molecular weight antigens (as high as 60 KDa) with both monoclonal as well as polyclonal antibodies. The same group very recently used a Re(I) complex as a fluorophore which shows a long average life time of 2.7 μ s when bound to human serum albumin [91]. The compound has the potential use for high molecular weight analytes (10²-10⁵ KDa). However, low quantum yields and low extinction coefficients make

these metal to ligand charge transfer (MLCT) complexes less favorable for routine studies [92].

The tracer purity is very critical in the assay development for this technique. Many times the background fluorescence causes problems in determining the exact amount of an analyte. A good and reliable instrument is definitely a requirement as well as a good chemist.

3. Instrumentation

Although early fluorescence polarization instruments lacked sensitivity and were expensive and bulky, current instruments are compact and combine high sensitivity, low cost, high speed and resolution, and ease of use. To the best of our knowledge, Abbott Laboratories began producing the first automated fluorescence polarization instrument for the clinical laboratory (TDx) with test kits for various uses in human diagnostics [16]. However, the instrument is not available to the research community. Recent developments in instrumentation (Jolley Consulting and Research Inc., Panvera Corporation and others) have solved the early problems so that relatively simple and inexpensive instruments with high sensitivity are now widely available.

Conclusion

Fluorescence polarization, with its new developments in instrumentation and reagents, is a powerful but simple technique for routine, in house and field diagnostic applications for various viral and infectious diseases in humans as well as animals, therapeutic drug monitoring, detection of drugs of abuse, food born pathogens, grain mycotoxins and environmental monitoring of pesticides. This technique detects the change in molecular size or binding of various molecules during a reaction. The reactions are homogeneous, rapid and single step and require only one or two reagents beside the sample. Most of the reagents are very stable and the results are independent of intensity. Results are fast, accurate and free of contamination problems. Because only a few reagents are required, the assays are easy to develop and reagents can be stored easily. Instruments are relatively inexpensive, stable and suitable for field testing and automation. There are no washing steps, and the assays tolerate the use of relatively impure receptors, cloudy and colored solutions. It has also found a place in high throughput screening for drug discovery. These features make fluorescence polarization a useful adjunct to existing methodologies due to its ease of use, sensitivity, speed and cost effectiveness.

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