Fluorescence Polarization (FP)

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Principles

Fluorescence polarization (FP) measurements are based on the assessment of the rotational motions of species. FP can be considered a competition between the molecular motion and the lifetime of fluorophores in solution. If linear polarized light is used to excite an ensemble of fluorophores only those fluorophores, aligned with the plane of polarization will be excited. There are two scenarios for the emission.

Provided the fluorescence lifetime of the excited fluorescent probe is much longer than the rotational correlation time θ of the molecule it is bound to ($\tau >> \vartheta$) (θ is a parameter that describes how fast a molecule tumbles in solution), the molecules will randomize in solution during the process of emission, and, as a result, the emitted light of the fluorescent probe will be depolarized. If the fluorescence lifetime of the fluorophore is much shorter than the rotational correlation time θ ($\tau << \vartheta$) the excited molecules will stay aligned during the process of emission and as a result the emission will be polarized.

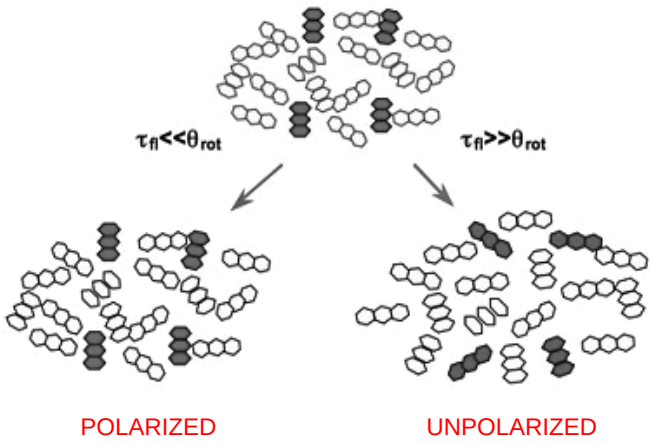

Figure 1. Relationship between fluorescence lifetime τ and rotational correlation time θ.

Dependence of Fluorescence Polarization on Molecular Mobility [1]

The fluorescence polarization (P) of a labeled macromolecule depends on the fluorescence lifetime (τ) and the rotational correlation time (θ)

$$\left(\frac{1}{P}-\frac{1}{3}\right)=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{\tau}{\theta}\right) \qquad [1]$$

where P_0 is the polarization observed in the absence of rotational diffusion. The effect of the molecular weight on the polarization values can be seen from an alternative form of the above equation:

$$\left(\frac{1}{P}-\frac{1}{3}\right)=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{kT}{\eta V}\tau\right) \qquad [2]$$

where k is the Boltzman constant, T is the absolute temperature, η the viscosity and V the molecular volume [2]. The molecular volume of the protein is related to the molecular weight (MW) and the rotational correlation time as given by

$$\theta=\frac{\eta V}{kT}=\frac{\eta MW}{RT}\left(\bar{\upsilon}+h\right) \qquad [3]$$

where R is the ideal gas constant, $\bar{\upsilon}$ is the specific volume of the protein and h is the hydration, typically 0.2 g H_2O per gram of protein. Generally, the observed correlation times are about two- fold longer than calculated for an anhydrous sphere due to the effects of hydration and the non- spherical shapes of most proteins. Hence, in aqueous solution at 20℃ ($\eta=1cP$) one can expect a protein such as HSA (MW ~ 65,000, with h = 1.9) to display a rotational correlation time $\theta\approx 50\,ns$.

The measurement of fluorescence polarization is relatively straight-forward (Figure 2). In a typical experiment the sample containing the fluorescent probe is excited with linear polarized light and the vertical and horizontal components of the intensity of the emitted light are measured and the polarization P or anisotropy r are calculated using the following equations:

$$P=\frac{I_V-I_H}{I_V+I_H} \qquad r=\frac{I_V-I_H}{I_V+2I_H} \qquad [4]$$

where I_V is the intensity parallel to the excitation plane and I_H is the emission perpendicular to the excitation plane. They are interchangeable quantities and only differ in their normalization. Polarization P ranges from –0.33 to +0.5 while the range for anisotropy r is –0.25 to +0.4. When the polarization is known, one can calculate the anisotropy, and viceversa, using the following relations:

$$P=\frac{3r}{2+r} \qquad r=\frac{2P}{3-P} \qquad [5]$$

Figure 2 shows a schematics of a typical instrument for the measurement of the polarization. The light from the source goes through a polarizer before impinging on the sample. The fluorescence is collected at 90 degrees through a movable polarizer located in the emission channel of the instrument.

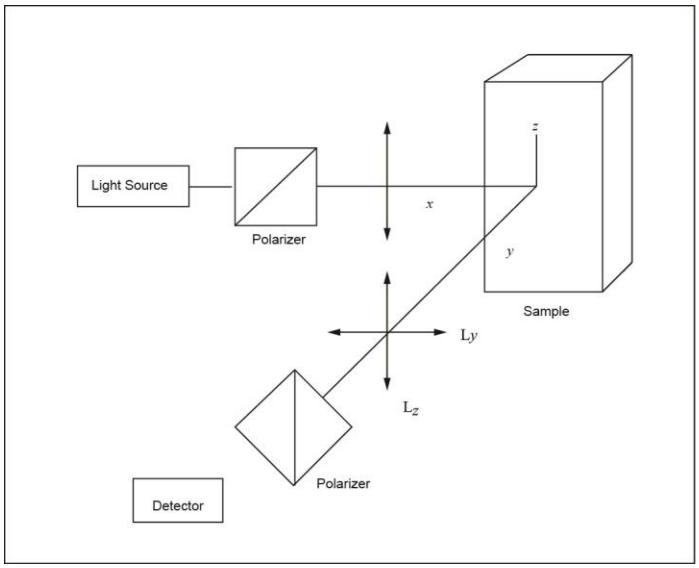

Figure 2. Schematic drawing for the measurement of fluorescence polarization.

Tracers for Polarization Assays

Tracers used in fluorescence polarization assays include peptides, drugs, antibiotics etc. and they are typically synthesized by the reaction of a fluorescent dye with a reactive derivative of the analyte.

Linker chemistries can have an impact on the fluorescence polarization. While tracers with short linkers between the fluorophore and the labeled molecule minimize the “propeller-effect”, a too short linker can affect the binding affinity of the tracer [3].

Typical fluorophores used in FP are fluorescein and rhodamines. BODIPY dyes have longer excited-state lifetimes than fluorescein and rhodamine dyes, making their fluorescence polarization sensitive to binding interactions over a larger molecular weight range [4].

A limitation of current fluorescence polarization immunoassays (FPIs) is that they are useful only for measurement of low molecular weight antigens. This limitation is the result of the use of fluorophores, such as fluorescein, which display lifetimes near 4 ns. An FPI requires that the emission from the unbound labeled antigen be depolarized, so that an increase in polarization may be observed upon binding to antibody. For depolarization to occur the antigen must display a rotational correlation time much shorter than 4 ns, which limits the FPI to antigens with molecular weight less than several thousand Daltons.

A class of dyes that have been shown to combine long lifetime and high polarization are the metal-ligand complexes of Ru, Os and Re. These labels have lifetimes in the range of a few hundred ns to microseconds and would therefore allow measurement of higher molecular weight antigens but the strong propeller effect of the MLC when labeled to proteins other than HSA has limited their use as labels for high molecular weight analytes [2].

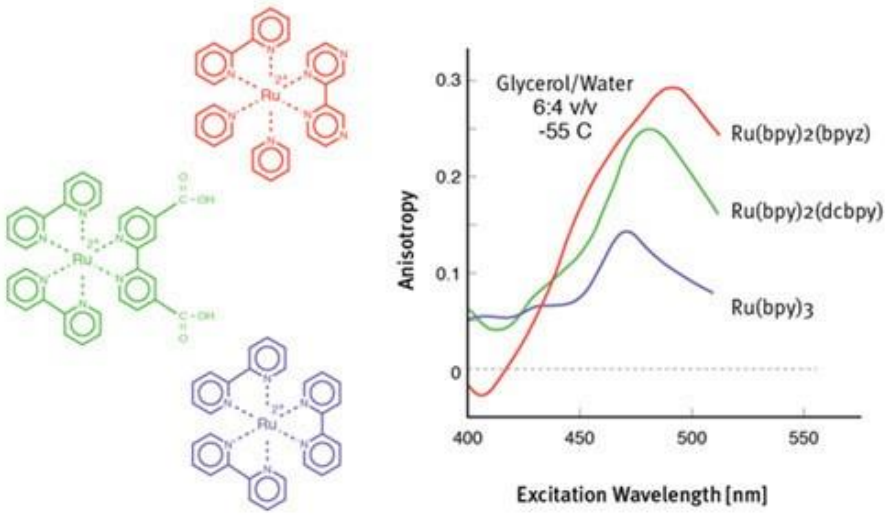

Figure 3. Excitation polarization spectra of Ru-metal ligand complexes in solution at -55℃.

Applications

Fluorescence polarization measurements have been used in analytical and clinical chemistry [5,6] and as a biophysical research tool for studying membrane lipid mobility [7], domain motions in proteins, and interactions at the molecular level [8]. Fluorescence polarization based immunoassays are also extensively utilized for clinical diagnostics [9-11].

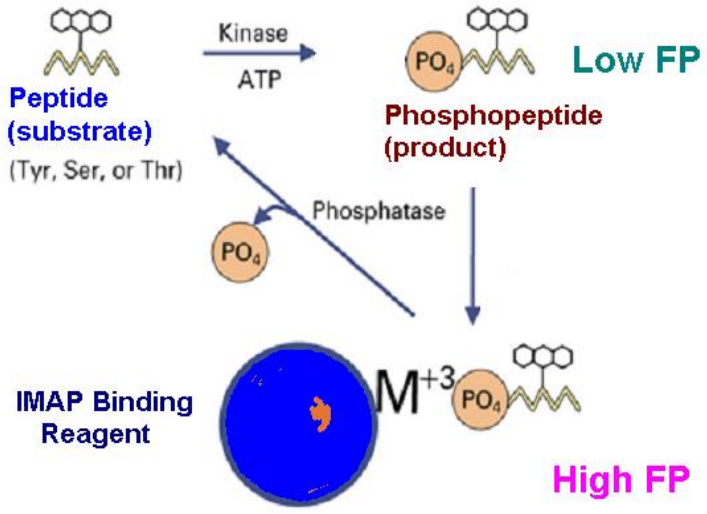

Figure 4. IMAPTM homogeneous binding assay for kinases and phosphatases.

FP has the advantage that it requires only one labeled species for the assay (unlike energy-transfer based read outs that require two labeled species) and thus FP has become a very popular read out format for HTS (12-17). Many of these assays are based on the use of antibodies that provide the specificity needed to selectively detect a wide variety of antigens.

An example for a homogeneous binding assay based on FP is shown below. Any material that enables a mass-increase of the labeled species can replace antibodies. In the IMAP assay™ (Molecular Devices) the high affinity of trivalent metal-ions to phosphate is utilized to generate the FP read-out [18].

Books and Book Chapters related to Fluorescence Polarization

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2. *Ichinose, N., Schwedt, G., Schnepel, F.M. and Adachi, K. (Eds.) (1987). Fluorometric Analysis in Biomedical Chemistry, J. Wiley & Sons, New York.*
3. *Van Dyke, K. and Van Dyke, R. (Eds.) (1990). Luminescence Immunoassay and Molecular Applications, CRC Press, Boca Raton, FL.*
4. *Lakowicz, J.R. (1999). Principles of Fluorescence Spectroscopy, 2nd Edition, Kluwer Academic/Plenum Publishers, New York.*
5. *Steiner, R. F. (1991). Fluorescence anisotropy: theory and applications. In Topics in Fluorescence Spectroscopy. Vol. 2. Principles. Lakowicz, J.R. (Ed.) Plenum Press, New York.*

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13. *Kim et al. "Development of a Fluorescence Polarization Assay for the Molecular Chaperone Hsp90". J Biomol Screen 9, 375-381 (2004).*

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15. *Turek TC, Small EC, Bryant RW et al. "Development and validation of a competitive AKT serine/threonine kinase fluorescence polarization assay using product-specific antiphospho-serine antibody". Anal. Biochem. 299, 45–53 (2001).*

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