

FLUORESCENCE POLARIZATION ASSAYS

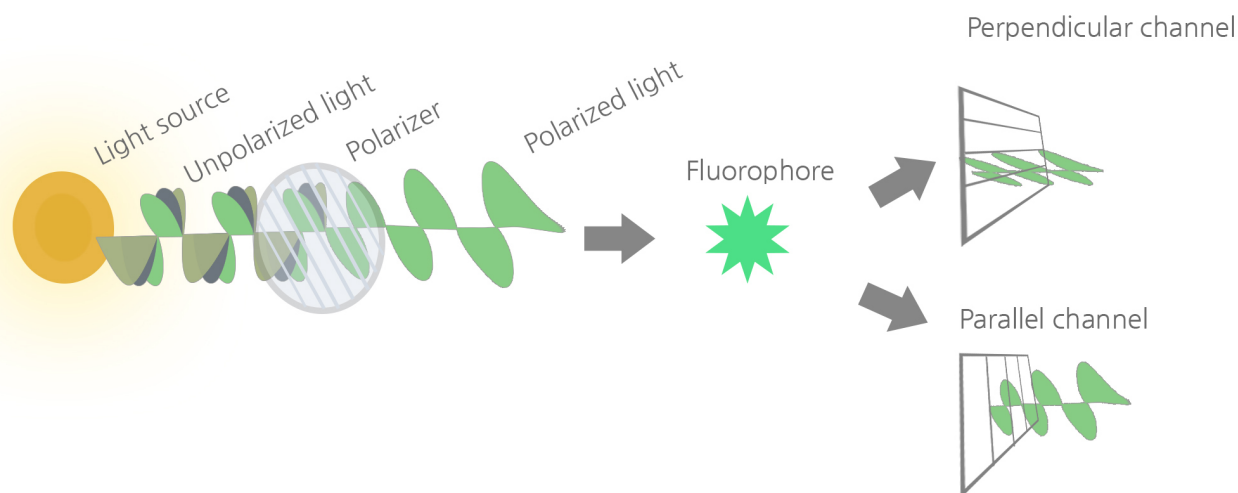
Introduction

Fluorescence Polarization (FP) technology measures changes in light polarization emitted by a fluorescent tracer in a sample and is quite different from fluorescence intensity, which measures the intensity of emitted light at a specific wavelength. FP is widely used to monitor molecular interactions in solution and provides a basis for direct and competition assays. FP assays are easily amenable to high-throughput formats, making them particularly useful for screening applications. FP is a complex technique that requires careful design and uses a specific instrument (a plate reader with fluorescence filters capable of polarized light excitation and capture of emitted light on two planes). This note explores the principles underlying the technology and FP-based experiments.

Principle of FP

In FP technology, the fluorescent dye is excited by polarized light. Although the initial light source

emits light in all directions, a polarizer filters the light and limits it to a single plane along the direction of propagation (Figure 1). When a fluorescent dye is excited by the polarized (single-plane) light, it re-emits light in all directions, because it moves around and rotates between the time of excitation and the time of emission (i.e., it depolarizes the excitation light). The molecular rotation is due to Brownian movement, which happens within nanoseconds, and the extent of light depolarization is proportional to this movement/rotation. Indeed, Dr. Weigert observed in 1920 that the polarization of the light re-emitted by a fluorophore decreases as increasing temperature accelerates Brownian movement, whereas it increases as high solvent viscosity slows the movement of the fluorophore. Similarly, movement decreases with the size of the fluorophore, and this increases polarization (history of FP and theoretical foundations reviewed in [1]).



Thus, the degree of light polarization of a fluorescent probe is inversely proportional to Brownian movements. Consequently, FP is affected by all parameters that alter the rotation and random movement of a molecule such as size, shape (a sphere rotates faster), solvent viscosity and temperature. When temperature and viscosity are kept constant, size becomes the main factor driving FP, which is then directly proportional to the size of the fluorescent probe.

To summarize, the degree of polarization of a fluorescent probe is a term that indicates to what extent the light of excitation remains polarized. When a small fluorophore is excited by polarized light, its movement is fast and it re-emits light in all directions. Therefore, it depolarizes the excitation light and the degree of polarization is low. On the contrary, if a large fluorophore is excited by polarized light, reorientation is limited because movement is slow, the light remains mostly polarized at re-emission and the degree of polarization is high.

Experimentally, FP technology measures fluorescence intensity emitted by the fluorophore in the two planes of light that are parallel and perpendicular relative to the plane of excitation. The degree of fluorescence polarization (P) is defined as the difference between the fluorescence intensity parallel and perpendicular relative to the plane of excitation, divided by the total fluorescence intensity:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

I_{\parallel} = Fluorescence Intensity parallel to plane of excitation

I_{\perp} = Fluorescence Intensity perpendicular to plane of excitation

Most instruments display fluorescence polarization in units of mP in which 1 mP = 1000 P

$$mP = \left(\frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \right) \times 1000$$

Since (P) is a ratio of light intensities, it is a number without dimension. Theoretical (P) values range from -0.33 to 0.5 (-330 to 500 mP). Experimental data typically range from 10 mP to around 300 mP. Instruments achieve very precise measurements ($P \pm 0.002$ or ± 2 mP).

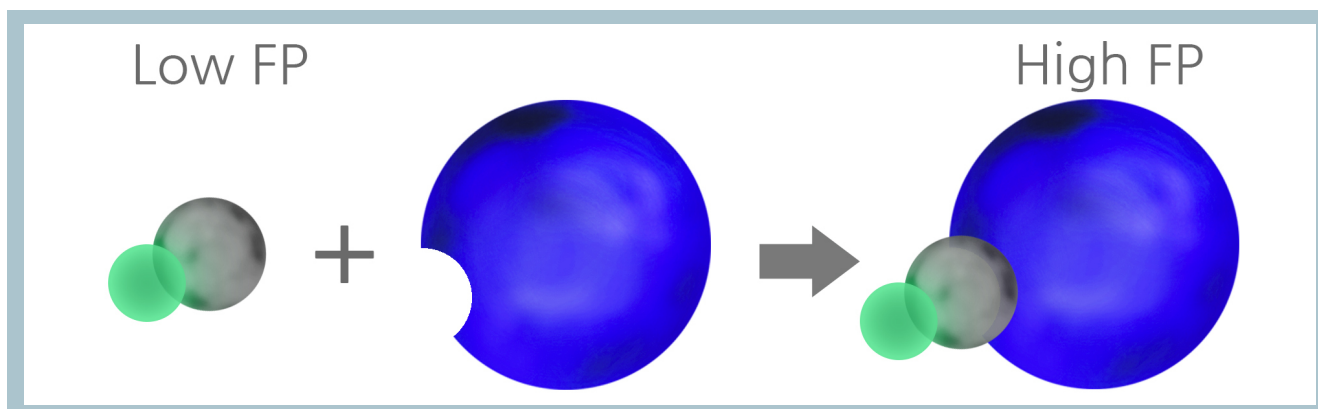
The equation above assumes that light is transmitted equally well through both parallel and perpendicular channels. In practice, this is not true and a correction must be made. The correction factor is called the "G Factor".

$$mP = \left(\frac{I_{\parallel} - G(I_{\perp})}{I_{\parallel} + G(I_{\perp})} \right) \times 1000$$

OR

$$mP = \left(\frac{G(I_{\parallel}) - I_{\perp}}{G(I_{\parallel}) + I_{\perp}} \right) \times 1000$$

The G-factor is instrument-dependent and needs to be determined by the investigator. The instrument manual will contain information about how to establish the G-factor; see also our [FAQ](#).



FP Assays

FP assays take advantage of the fact that the depolarizing property of a fluorophore depends on its size by measuring changes in light polarization that occur when a small fluorophore interacts with, or dissociates from, a much larger partner. Therefore, FP experiments detect dynamic interactions in which a fluorescent tracer transitions from low polarization to high polarization forms because of changes in size consequent to the reaction under study.

Any small molecule, typically less than or around 2 kDa, which can be covalently labeled with a fluorophore and can form a complex with a larger partner is amenable to an FP assay.

Example: Upon binding to a much larger partner, the rotation of a small fluorophore becomes limited, speed of reorientation decreases significantly, and when excited with polarized light it now emits a mostly polarized light during its excitation state.

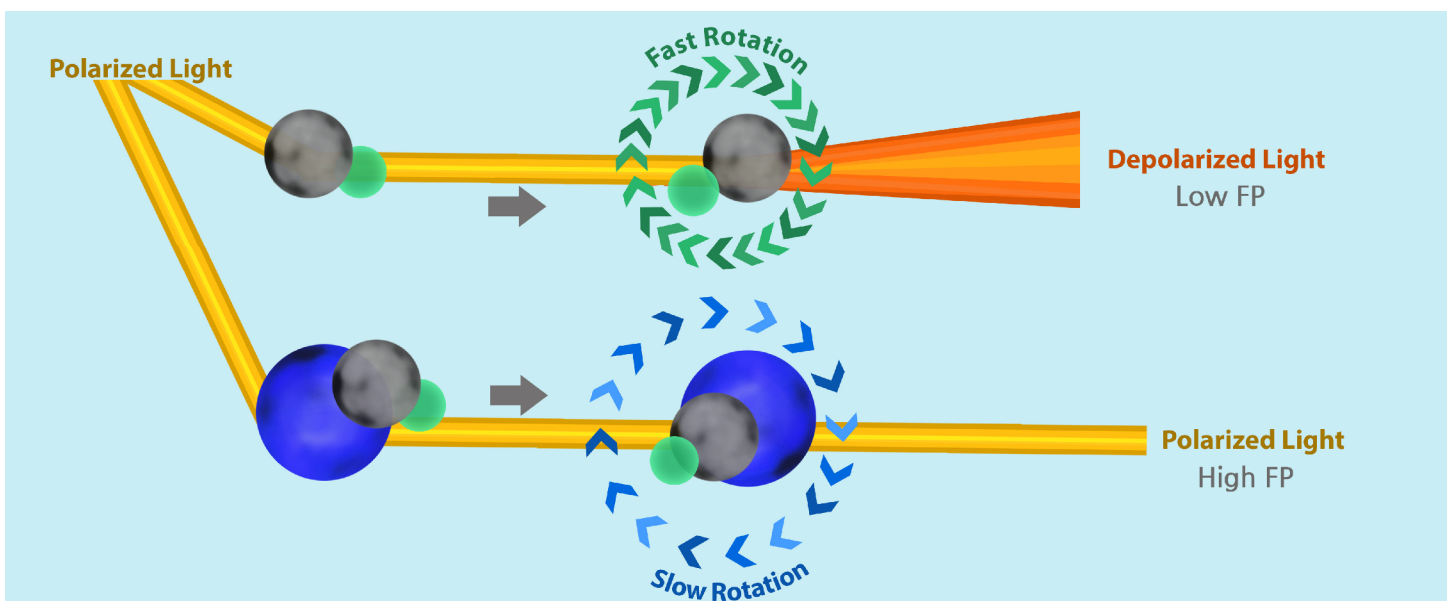
Several controls are necessary to conduct a successful experiment:

- "Blank" contains buffers and solvent but does not contain the tracer or the binding partner.

The "Blank" takes into account the small auto-fluorescence that may come from the assay buffers, and should be lower than the "Reference". It is subtracted from all measurements.

- "Reference" is an internal control that contains the tracer but does not contain the binding partner. It is the lowest FP allowed by the experimental conditions, representing the condition in which all of the small fluorescent tracers are present in free form.
- "High FP" is an internal control corresponding to the highest FP allowed by the experimental conditions, in which most or all the available tracer is in its bound state. In many (but not all) types of assays, this is the same as the "Positive control".
- "Positive control" is the experimental control. For example, if an inhibitor of the interaction is being tested, the positive control is the condition without the inhibitor.

Depending on the experimental setting and type of assay, the "high FP" control can be omitted if it is the same as the positive control.



Applications

FP can be used in any experiment that involves the molecular binding of two entities where one is much smaller than the other, as long as the smaller entity can be fluorescently labeled [2]. It is rather straightforward to establish a competition

or an inhibition assay; proteolysis and other enzymatic assays have been developed as well. A general procedure for the development of FP assays can be found in [3]. Examples abound in which FP assays have been used successfully:

- Binding of an antibody to a small antigen (epitope)
- Receptor-ligand interaction (e.g. growth factors and cytokines)
- Protein-protein interaction
- Protein-DNA and protein-RNA interaction
- Enzymatic reaction: substrate binding to the enzyme or formation of a new product
- Proteolysis
- Formation of a new product that is distinguished using specific fluorescent-beads. For example, measure of phosphorylation using a fluorescent bead that is specific for the phosphorylated product
- Detection of specific PCR products [4]
- SNP detection by allele-specific primer extension
- Competition studies in which the tracer is displaced, determination of EC50
- High-throughput screening of small molecule inhibitors
- Study of membrane lipid mobility
- Screening for inhibitors of alpha-synuclein oligomerization [5]
- Study of muscle function [6]

Advantages and Limitations

Although FP assays are complex to develop, a well-designed assay is simple to use and highly amenable to high-throughput formats. Here are a few characteristics that make FP assays particularly attractive:

Advantages

- In-solution
- Tolerate very small volumes
- Homogenous, no wash steps
- Non-radioactive
- Real-time
- Polarization-based readouts are somewhat less dye dependent and less susceptible to environmental interferences such as pH changes than assays based on measurement of fluorescence intensity

Limitations

- No kinetic constants
- The fluorescent tag, if not properly designed, may alter the binding properties of the tracer. Careful validation is highly recommended
- Sensitivity to changes in temperature

Considerations

Factors that do not interfere significantly

- FP assays are not sensitive to buffer pH
- Polarization does not depend on the concentration of dye, and variations in fluorescence intensity due to the presence of color additives produce relatively minor interference

Critical factors

- A change in size is the most critical factor in FP assays; ideally by a factor of at least 5 times (e.g: a 2 kDa fluorescent tracer binding to a 10 kDa protein or larger). The higher the difference, the more robust the measurements will be
- Fluorescent molecules have an excited state, and the lifetime of this excited state influences FP, since the longer the molecule remains excited the more it rotates. This does not affect measurements as it is an intrinsic property of the fluorophore and it remains constant throughout the experiment. However, it influences the choice of fluorophore since more stable fluorophores allow for more robust measurements
- Performance of an assay depends on the extent to which the biological activity of the small tracer is disrupted by the labeling. The choice of the dye used for labeling is an important factor. Validation of the fluorescent tracer is critical to ensure that labeling does

not alter its interaction with the molecule of interest, or does not affect the enzymatic reaction under study

- The quality of the fluorescent labeling is also critical: the tracer should be >90 % labeled and the free dye should be eliminated. If a high percentage of the tracer is not labeled, it will compete with the labeled tracer for binding to the partner, which will alter the apparent IC50. If the free dye is not eliminated, it will result in a high background that will decrease the robustness of the assay
- The purity and quality of the components influences the quality of the assay. Potential interference in light scattering can be caused by many large molecules such as cell or membrane debris, therefore the partner protein needs to be pure. For this reason crude cell lysates, cell culture supernatants and other rudimentary extracts should not be used. The presence of contaminants with high background fluorescence, or large contaminants with non-specific trapping ability such as BSA (bovine serum albumin), is likely to result in high noise-to-signal ratios or to otherwise interfere with the signal, which will decrease sensitivity. Cleanliness of glass and plastic vessels, absence of contamination in the buffers are all important factors when performing FP assays

Conclusion

BPS Bioscience offers validated FP-based assay kits, saving scientists time and money by eliminating the need for many steps of optimization such as design, labeling and validation of the fluorescent tracer, or improvement of the assay conditions. Our kits provide high quality purified proteins and are provided with a validated protocol.

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