AppliedPhotophysics
Ultrasensitive Spectroscopy for the Life Sciences

Abstract: Stopped-flow fluorescence polarisation/anisotropy is a highly useful technique for obtaining valuable kinetic information regarding biomolecular interactions. The technique may be used to measure the rates of structural changes which can therefore offer significant advantages over conventional fluorescence intensity experiments.

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SX SERIES APPLICATION NOTE

Stopped-Flow Fluorescence Polarisation/Anisotropy

Fluorescence Polarisation-Stopped-flow is a useful technique that enables the acquisition of fluorescence polarisation/anisotropy kinetic data. The technique can be applied to study many types of biomolecular interactions, including Protein-Ligand interactions, Protein-Protein interactions, and Protein-DNA interactions. This review describes the basic principle of the FP technique along with a specific example of how the accessory can be applied in research to obtain insightful information.

THEORY

Absorption of an incident photon by a molecule may only occur when the molecule's transition dipole moment is aligned with the electric vector of the incident light. In a solution, the orientations of transition dipole moments are randomly distributed. If unpolarised light (such that of a standard SX20 Xenon arc lamp) were incident on a solution of fluorophore, light would be absorbed equally in all orientations due to the random orientations of both the radiation and the molecules. Consequently, fluorescent light will be emitted at all angles in equal intensity. If however the light incident on the solution is polarised in a particular plane, only molecules whose transition dipole moments are parallel to that plane will absorb the incident light.

KEYWORDS

▸ Stopped-flow	▸ SX20
▸ Polarisation	▸ Rapid mixing
▸ Anisotropy	▸ Reaction kinetics
▸ Biomolecular Interactions	▸ Dual Fluorescence

Assuming (incorrectly) that the molecules were stationary, the emitted light will be polarised in the orientation of the molecule's emission dipole moment. In a real solution however, some of this polarisation will be lost, largely be due to rotational diffusion (or "tumbling") of the excited molecule. The extent of this loss of polarisation will mostly depend on two factors: The lifetime of the excited state and the speed of rotation of the fluorophore. The latter of these implies that slowly rotating molecules will exhibit a higher degree of polarisation than those that rotate quickly. It is this principle that can be exploited using the FP accessory. The principle of the technique is summarised in Figure 1.

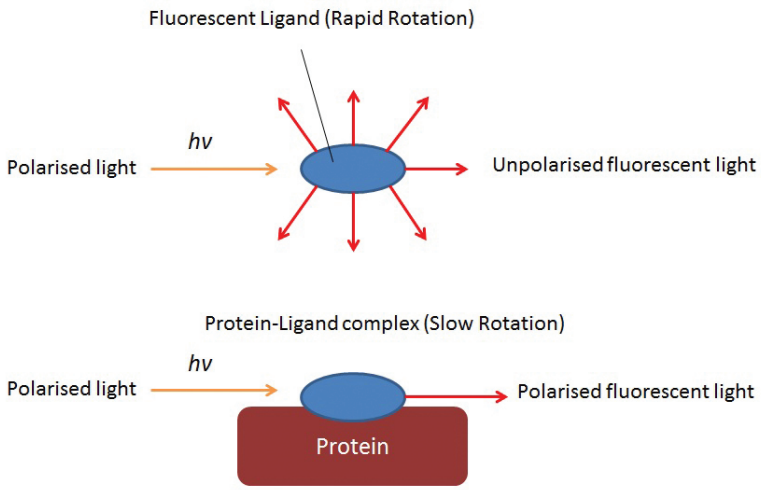

Figure 1. Principle behind fluorescence polarisation/anisotropy experiments with regard to Protein-Ligand binding. When the Ligand is free in solution it has a high rate of rotational diffusion and will therefore apparently emit unpolarised light. When the Ligand is Protein-bound, its rotational diffusion rate is significantly decreased resulting in an observed increase in polarisation of the emitted light.

For example, the fluorescent light from a protein binding drug will be more polarised when bound to a protein than when free in solution. This allows the kinetics of the binding interaction to be probed because we can observe the bound and unbound states of the molecule.[1]

Polarisation (*P*) is given by equation (1):

$$P = \frac{(I_{II} - I_{\perp})}{(I_{II} + I_{\perp})} \quad (1)$$

Where I_{II} is equal to the intensity of light emitted parallel to the plane of polarised excitation light and $I_{\perp}$ is equal to the intensity of light emitted perpendicular to the plane of polarised excitation light.

Commonly, *P* is expressed as Anisotropy, (*A*); mathematically these two quantities are interchangeable. *A* is given by equation (2)

$$A = \frac{(I_{II} - I_{\perp})}{(I_{II} + 2I_{\perp})} \quad (2)$$

Measuring the fluorescence anisotropy can offer several benefits over just measuring the fluoresence intensity. This is because anisotropy can give structural details relating to the environment of the fluorophore (i.e. how has the mobility of the fluorophore changed). Additionally, anisotropy can be measured in interactions that do not exhibit a change in fluoresence intensity.[2]

To use an SX stopped-flow to measure *A*, an "excitation assembly" converts the unpolarised light into light that is polarised in a user defined orientation. Two photomultiplier tube (PMT) detectors are placed at right angles to the incident polarised radiation with a polariser located in front of each PMT; the polarisers are arranged so that one PMT detects emitted light parallel to the excitation beam and the other detects emitted light perpendicular to excitation beam. This arrangement is displayed in Figure 2.

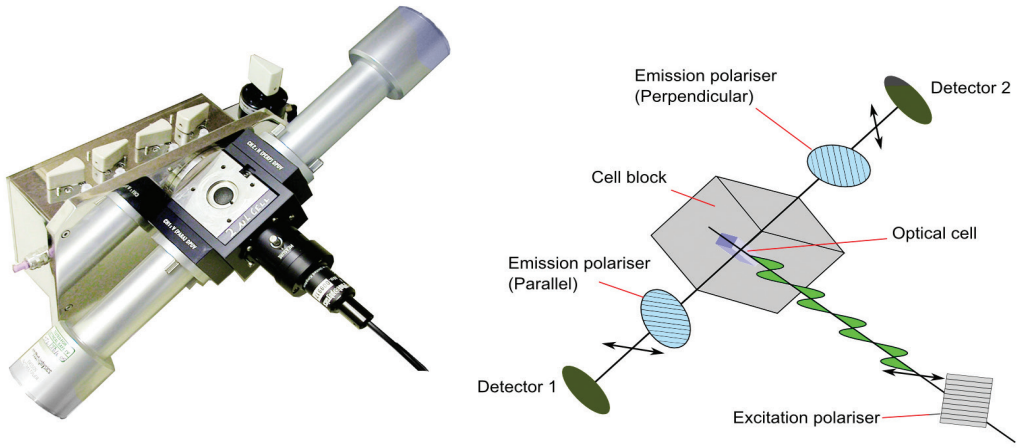

Figure 2. Arrangement of the FP accessory of an SX stopped-flow spectrometer.

In order to correct for gain differences in the two detection channels, it is necessary to determine a G-factor prior to running the experiment. G-factor determination is controlled from the SX software and both kinetics and spectra may be acquired in polarisation, anisotropy, and other modes with full post-acquisition conversions available as required. When taking the G-factor into account, equation (2) becomes:

$$A = \frac{(GI_{\parallel} - I_{\perp})}{(GI_{\parallel} + 2I_{\perp})} \qquad (3)$$

Given that the tumbling rate will be dependent on the viscosity of the solvent, an increase in anisotropy of a fluorophore is observed when the viscosity of its solvent is increased. Figure 3 shows the anisotropy of a 3μM solution of fluorescein with increasing glycerol concentrations measured in an SX20 using the FP accessory.

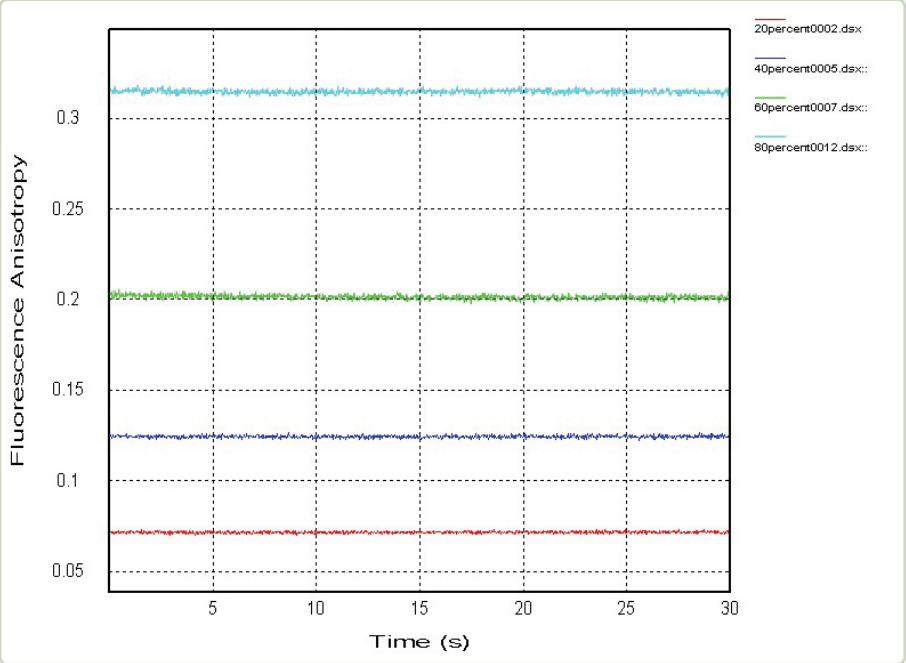

Figure 3. Single traces obtained when measuring the anisotropy of 3μM fluorescein solutions in increasing glycerol concentrations- Red line = 20%, Blue line = 40% Green line = 60%, Light Blue line= 80% (V/V).

SELECTED EXAMPLE FROM SCIENTIFIC LITERATURE

Reference[3]: Henn, A. et al. Pathway of ATP utilization and duplex rRNA unwinding by the DEAD-box helicase, DbpA. Proceedings of the National Academy of Sciences of the United States of America 107, 4046-50 (2010).

One of the valuable uses of this type of system is to study the interactions of proteins with nucleotides. An excellent example of this method is described in reference[3]. This piece of research was studying the behaviour of DbpA, a 'DEAD-box RNA helicase'. These proteins act to rearrange the structure RNA molecules using energy from ATP hydrolysis.[4]

DbpA from *E.coli* has been shown to specifically bind near hairpin 92 of 23S from *E.coli* (a ribosomal RNA molecule). Only a small portion of the RNA molecule is required for this specific interaction, provided the RNA possesses the hairpin 92 connected to the Helix 91 sequence. (Figure 4 shows an example of such a complex; this is high resolution X-ray structure of the C-terminal part of DbpA from Bacillus subtilis bound to part of 23S from *E.coli*)[5]. DpbA has been shown to unwind bound duplex RNA strands *in vitro* in the presence of ATP.[6]

Stopped-flow fluorescence polarisation analysed the DbpA catalysed hydrolysis of a duplex RNA molecule in the presence of ATP. The RNA duplex consisted of a 32-mer strand base paired to an 8-mer strand. The 32-mer strand contains the hairpin structure (hairpin 92 of 23S in *E.coli*) connected to the Helix 91 sequence. The 8-mer strand is covalently linked to a fluorescent molecule; in this case fluorescein (Figure 4). The duplex structure binds to the DbpA creating a DpbA-dsRNA complex (As shown in Figure 4) which becomes unwound following ATP hydrolysis.

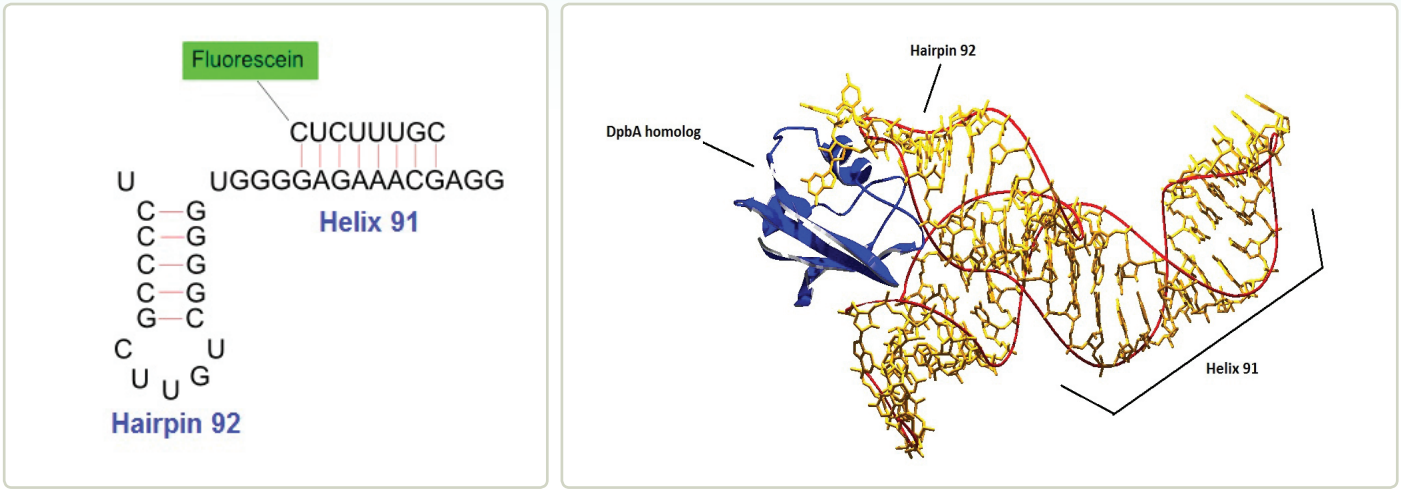

Figure 4. Left – Adapted from Reference[3]. Structure of the duplex RNA molecule used in the described study. Right – Structure of a DpbA-dsRNA complex determined in Reference [5].

The experiment was therefore conducted as follows: After equilibrating DbpA with the duplex RNA, the complex was rapidly mixed with ATP in an SX20 set up for measuring FP. A representation of the published data is displayed in Figure 5.

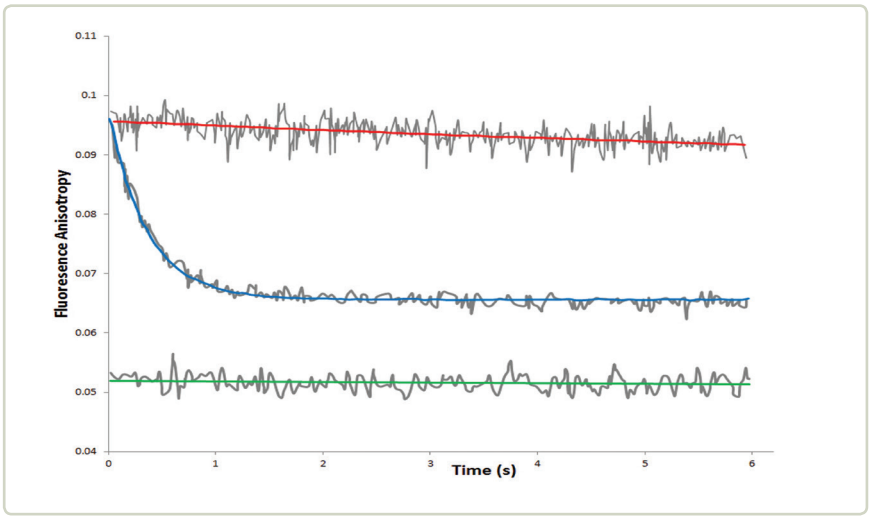

Figure 5. Representation of data obtained from Reference[3]. Changes in anisotropy when the DpbA-dsRNA complex reacted in a stopped flow with buffer (Red line), ATP (Blue line) and the Green line shows the anisotropy of the isolated 8-mer.

Figure 5 shows that there is a decrease in anisotropy over time. This is attributed to unwinding of the duplex RNA and release of the 8-mer strand. This makes intuitive sense in relation to the above discussion on anisotropy. The DpbA-dsRNA complex is a large macromolecule and will therefore be rotating slowly and thus the fluorophore retains the polarisation of the emission dipole moment.

On release of the 8-mer into solution, the fluorophore will be rotating faster, resulting in loss of polarisation. It is interesting to note that the anisotropy does not reduce to the level displayed by the same concentration of labelled 8-mer without DpbA. This is mostly attributed to re-annealing of the duplex.

This experiment was repeated with the 32-mer strand being labelled rather than the 8-mer. Under these conditions, no significant change in anisotropy was observed. This result indicates that the 32-mer remains bound to DpbA subsequent to dissociation of the other strand.[3] The study demonstrates the type of experiments that can be conducted using an SX instrument set up for measurement of fluorescence polarisation. It is clear that important structural information can be obtained by anisotropy measurements.

OBTAINING KINETIC PARAMETERS FROM ANISOTROPY MEASUREMENTS

In order to extract kinetic parameters (i.e. rate constants) from the type of data described above, it is necessary record the total intensity fluorescence (*S*) as well as the anisotropy. The Pro-Data software records this information simultaneously with anisotropy. This is given by equation (4):

$$S = (GI_{\parallel} - 2I_{\perp}) \quad (4)$$

In measuring *S*, the differences in fluorescence intensities of the species at the start and end of reaction can be accounted for. If there is no change in fluorescence intensity then the anisotropy data can be treated as any other type of kinetic trace. (Absorbance, Fluorescence etc.) If however the change in anisotropy is accompanied by an intensity change, treatment of the data becomes more complex.

$$A_{obs}(t) = \frac{(r_1 - r_2)}{(1-D)+De^{kt}} + r_2$$

For a first order reaction (single exponential decay) of species 1 to species 2, the observed anisotropy at time t, $A_{obs}(t)$ is then given by equation (4).

Where r_1 and r_2 represent the steady state anisotropies of species 1 and species 2 respectively. *D* is equal to the ratio of intensities of species 2 to species 1. k represents the first order rate constant. Fitting the data to this equation can therefore extract the value of the first order rate constant.

As expected, when there is no difference in intensity between the two species (i.e. $D = 1$), the anisotropy will follow first order kinetics.

For a more thorough discussion on how to fit kinetic anisotropy data, see Reference[7].

The fluorescence polarisation accessory for the SX stopped-flow series provides an excellent means for recording kinetic anisotropy measurements. This is useful for probing the details of molecular interactions. Applied Photophysics' proprietary Pro-Data software allows simple collection of kinetic anisotropy data in which the G-factor is automatically corrected for using a wizard in the Pro-Data software.

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