

Stopped-Flow Fluorescence Polarisation/Anisotropy

- Can be applied to study many types of biomolecular interactions, including protein-ligand interactions, protein-protein interactions, and protein-DNA interactions.
- Obtain important structural information by correlating fluorescence polarisation with substrate variation.
- Understand the basic theory behind fluorescence polarisation and how to measure it with a SX20.

Stopped-flow fluorescence polarisation/anisotropy is a highly useful technique for obtaining valuable kinetic information about biomolecular interactions and structural changes of biomolecules. An example from the literature illustrates how the SX20 Fluorescence Polarisation accessory is used to gain insight into the kinetics of the interaction between a helicase and its RNA binding partner.



KEYWORDS

- SX20
- Stopped-Flow
- Reaction Kinetics
- Biomolecular Interactions
- Polarisation
- Anisotropy
- Rapid Mixing
- Dual Fluorescence

Introduction

Fluorescence polarisation (FP)-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 application note describes the basic principle of FP and illustrates how it can be applied in research using the SX20 FP accessory.

Case Study

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 [1]. This study elucidated the behaviour of DbpA, a 'DEAD-box RNA helicase'. These proteins act to rearrange the structure of RNA molecules using energy from ATP hydrolysis [2]. 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

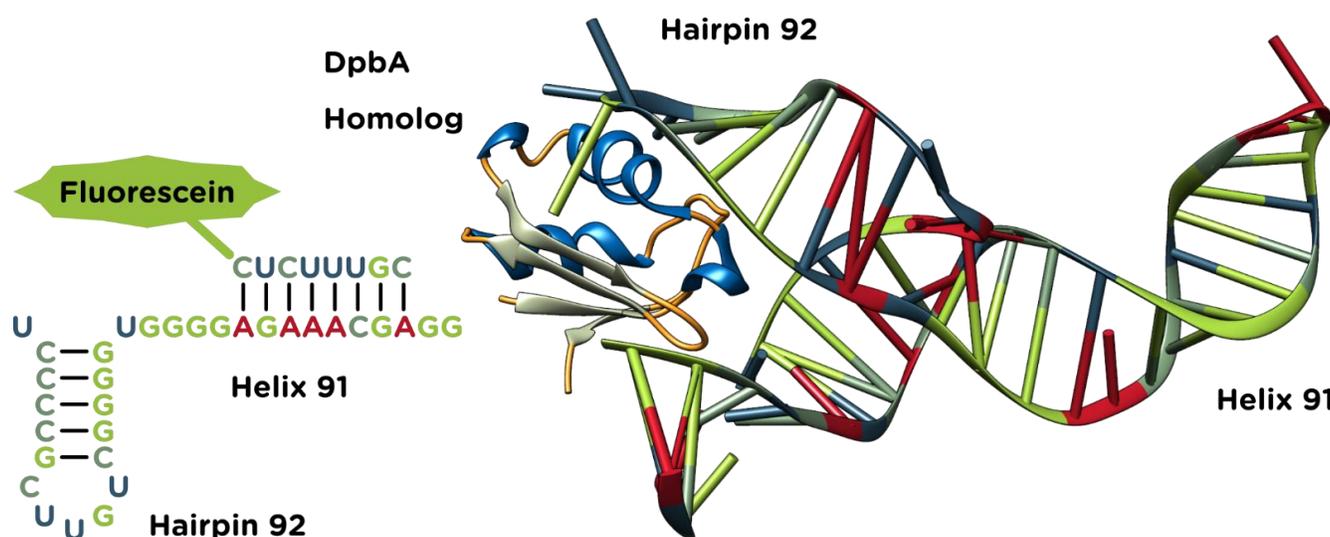


Figure 1: Structure of the duplex RNA molecule described (left, adapted from [1]) and structure of a DpbA-dsRNA complex consisting of the C-terminal part of DbpA from *Bacillus subtilis* and part of 23S from *E. coli* (right, adapted from [3], PDB ID: 3MOJ).

the RNA molecule is required for this specific interaction, provided the RNA possesses the hairpin 92 connected to the Helix 91 sequence. (Figure 1 shows an example of such a complex) [3]. DpbA has been shown to unwind bound duplex RNA strands *in vitro* in the presence of ATP [4].

The hydrolysis of a duplex RNA molecule catalysed by DpbA in the presence of ATP was analysed by stopped-flow fluorescence polarisation. 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, fluorescein (Figure 1). The duplex structure binds to DpbA creating a DpbA-dsRNA complex (Figure 1) which becomes unwound following ATP hydrolysis.

Results

Data published in [1] is shown in Figure 2 which 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 [1].

Conclusions

The study demonstrates the type of experiments that can be conducted using an SX20 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) from the type of data described above, it is necessary to record the total intensity fluorescence, S , as well as the anisotropy. The instrument control ("Pro-Data") software records this information simultaneously with anisotropy. This is given by equation 1:

$$S = (GI_{\parallel} - 2I_{\perp}) \quad (1)$$

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, 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.

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

$$r_{\text{obs}}(t) = \frac{(r_1 - r_2)}{(1 - D) + De^{kt}} + r_2 \quad (2)$$

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 [5].

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.

Experimental

After equilibrating DbpA with duplex RNA, the complex was rapidly mixed with ATP in an SX20 set up for measuring FP. Kinetic measurements were performed at 25°C. Fluorescein was excited at 485 nm and emission monitored at 515 nm.

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 5 (see box 'Fluorescence Polarisation/Anisotropy Basics') becomes:

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

For more experimental details, please refer to [1].

References

[1] A. Henn, W. Cao, N. Licciardello, S. E. Heitkamp, D. D. Hackney and E. M. De La Cruz, 2010, *Proc. Natl. Acad. Sci. USA*,

[2] O. Cordin, J. Banroques, N. K. Tanner and P. Linder, 2006, *Gene*, 367, 17-37.

[3] J. W. Hardin, Y. X. Hu and D. B. McKay, 2010, *J. Mol. Biol.*, 402, 412-427.

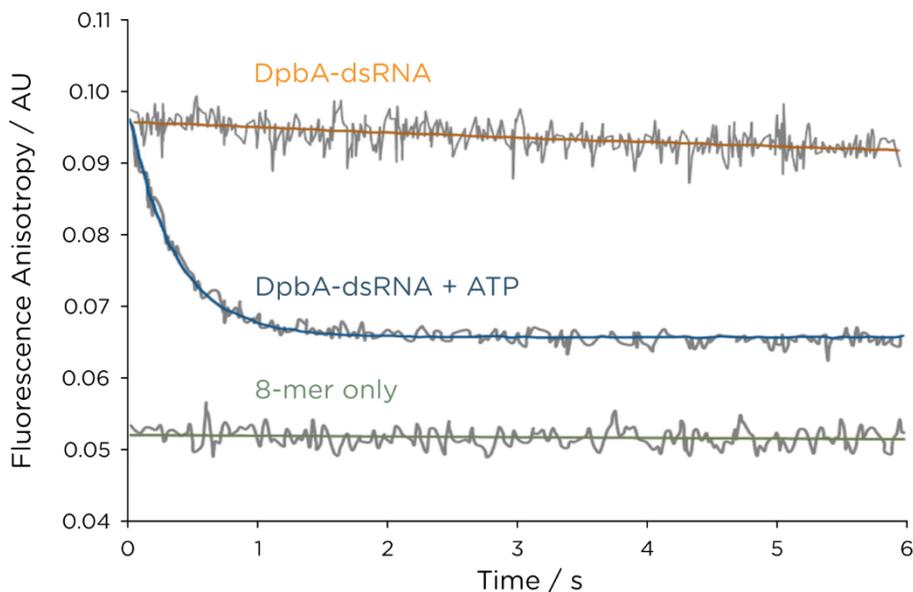


Figure 2: Changes in fluorescence anisotropy upon RNA hydrolysis catalysed by DpbA as monitored by stopped-flow.

[4] C. M. Diges and O. C. Uhlenbeck, 2001, *EMBO J.*, 20, 5503-5512.

[5] M. Otto and M. Lillo, 1994, *Biophys. J.*, 67, 2511-2521.

[6] J. R. Lakowicz, 1983, *Principles of Fluorescence Spectroscopy*.

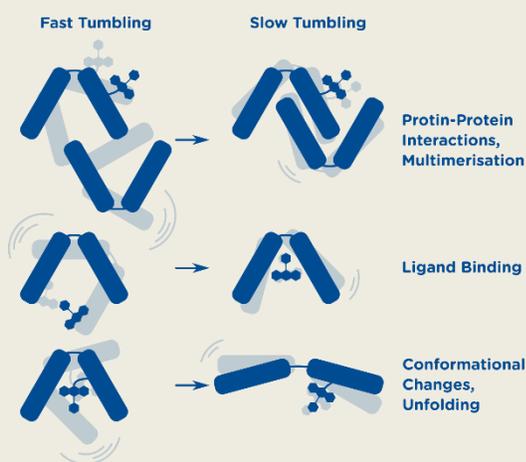
[7] J. F. Eccleston, 2001, *Oxford University Press*.

© Applied Photophysics Limited, 2019. All rights reserved. SX20™ is a trademark of Applied Photophysics Limited. All other trademarks are the property of their respective owners.

Fluorescence Polarisation/Anisotropy Basics

Absorption of an incident photon by a molecule only occurs when the molecule's transition dipole moment is aligned with the electric vector of the incident light. In solution, the orientations of transition dipole moments are randomly distributed. If unpolarised light (such as 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 would be emitted at all angles in equal intensity. If, however, the incident light is polarised in a particular plane, only molecules whose transition dipole moments are parallel to that plane will absorb the incident light.

If the molecules were stationary, the emitted light would be polarised in the orientation of the molecule's emission dipole moment. In a real solution however, some of this polarisation is lost, largely due to rotational diffusion (or "tumbling") of the excited molecules.

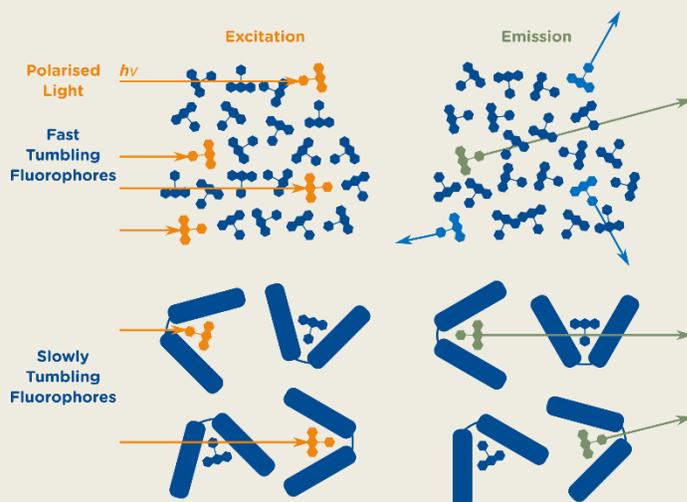


The extent of loss of polarisation due to molecule tumbling mostly depends on two factors: The lifetime of the excited state and the speed of rotation of the fluorophore. The latter implies that slowly rotating molecules exhibit a higher degree of polarisation than those that rotate quickly. It is this principle that can be exploited using the FP accessory.

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 [7]. Polarisation, P , is given by equation 4:

$$P = \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + I_{\perp})} \quad (4)$$

Where I_{\parallel} 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, Polarisation, P , is expressed as Anisotropy, r ; mathematically these two quantities are interchangeable. r is given by equation 5:

$$r = \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + 2I_{\perp})} \quad (5)$$

Measuring the fluorescence anisotropy can offer several benefits over just measuring the fluorescence 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 fluorescence intensity [6].

To use an SX stopped-flow to measure r , 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 the excitation beam.

