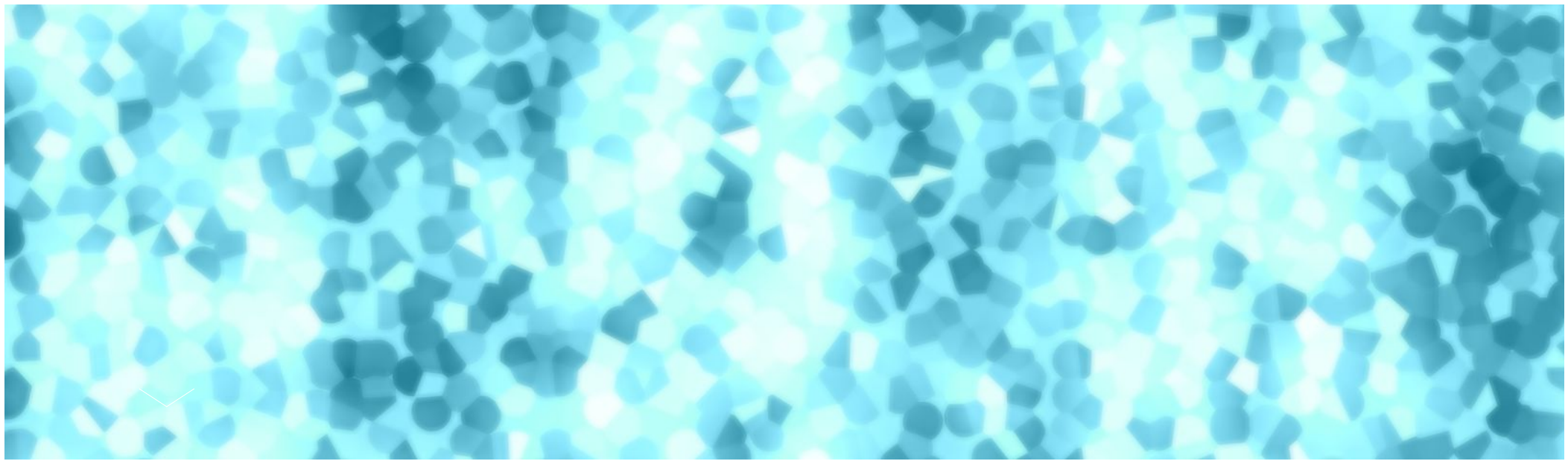

Fluorescence Spectroscopy

What is Fluorescence Anisotropy or Fluorescence Polarization?

Fluorescence anisotropy or fluorescence polarization is a measurement of the changing orientation of a molecule in space, with respect to the time between the absorption and emission events. Absorption and emission indicate the spatial alignment of the molecule's dipoles relative to the electric vector of the electromagnetic wave of excitation and emitted light, respectively. In other words, if the fluorophore population is excited with vertically polarized light, the emitted light will retain some of that polarization based on how fast it is rotating in solution. The faster the orientation motion, the more depolarized the emitted light will be. The slower the motion, the more the emitted light retains the polarization.

Anisotropy, r $r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$

- VV denotes vertical excitation, vertical emission
- VH denotes vertical excitation, horizontal

Fig. 11: Anisotropy equation

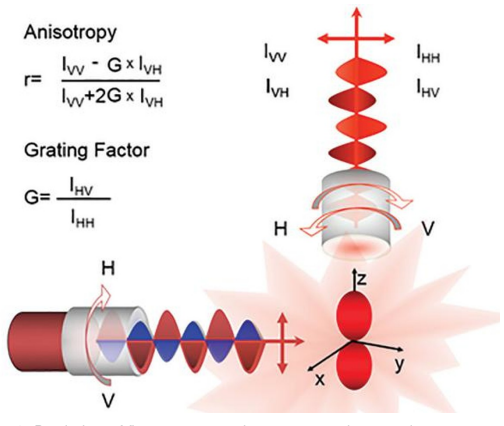

Fig. 12: Depiction of fluorescence anisotropy experiment using excitation and emission polarizers rotated to 0 degrees (Vertical, V) and 90 degrees (Horizontal, H) orientations.

To use this information, polarizers are placed in the excitation light path and the emission light path of a fluorometer. The anisotropy is calculated by taking the ratio of the intensities in the above equation, where IVV indicates the intensity with vertically polarized excitation and vertical polarization on the detected emission. IVH indicates the intensity when using a vertical polarizer on the excitation and horizontal polarizer on the emission. G is a grating factor used as a correction for the instrument's differential transmission of the two orthogonal vector orientations.

How does the experiment work? First the fluorescence is measured with the excitation polarizer set at vertical and the emission polarizer also set at vertical orientation. The intensity is plugged into the anisotropy equation as IVV. Then, the measurement is repeated with the emission polarizer set at horizontal orientation and the intensity for is put into the equation as IVH.

The anisotropy formula contains a factor of 2 because there are two orthogonal orientations the deflection from the VVz vector can be projected onto, Hx and Hy resulting in two IVH components.

A related expression, degree of polarization p is often used to describe a two dimensional polarization parameter with only one horizontal component accounted for. In this latter case the formula would lack the multiplier 2 for IVH with p taking the place of r.

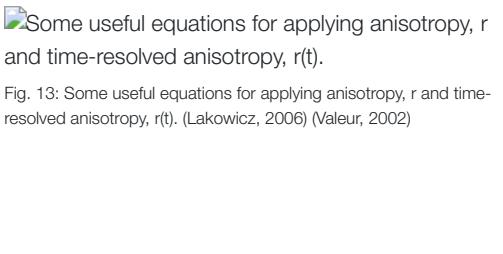

Fig. 13: Some useful equations for applying anisotropy, r and time-resolved anisotropy, r(t). (Lakowicz, 2006) (Valeur, 2002)

Next, the G-factor is calculated by measuring the intensity at HH and HV…and inserting IHV and IHH into the equation for G. Anisotropy, denoted by lower case "r" is often used as an indicator of molecular size, diffusion, and viscosity.

Fig. 14: Time-resolved anisotropy measurement

What are the applications of fluorescence anisotropy?

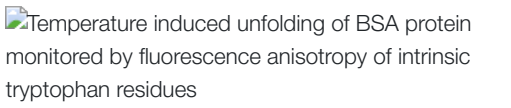

Fig. 15: Temperature induced unfolding of BSA protein monitored by fluorescence anisotropy of intrinsic tryptophan residues

Here are some equations useful for analyzing anisotropy results. The basic anisotropy equation has already been discussed, but the same can be calculated for entire fluorescence decays, giving time-resolved anisotropy. From time-resolved anisotropy, one can get reorientation time constants and then, use the Perrin equation and Stokes Einstein Debye equation to estimate properties such as diffusion coefficient, local viscosity, and molecular volumes.

These correspond to very important information when looking at applications such as protein or molecular binding, polymer aggregation, and other local environment studies in complex solutions and materials.

As one example, you can clearly see the temperature dependent protein unfolding behavior of BSA as measured by fluorescence anisotropy. The anisotropy of the intrinsic tryptophan residues are used in this case.

What are the uses of fluorescence kinetics?

Fluorescence kinetics refers to observation of fluorescence intensity over time. Here, a sample is excited at a single wavelength and the emission is detected at a single wavelength over time. Sometimes, wavelengths pairs are used for ratiometric dyes or to simultaneously record baseline or background information.

Reaction rates can be followed using time-based measurements as you can see here. In this example, the reaction rates of the binding of thiamine and mercury to form thiachrome are found by varying the concentration of thiamine used. Each kinetics scan represents a different reaction rate of the thiachrome formation reaction.

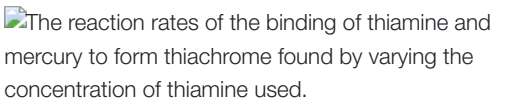
The reaction rates of the binding of thiamine and mercury to form thiachrome found by varying the concentration of thiamine used.

Fig. 16: Top left: Reaction of thiamine and Hg2+ to form thiachrome. Bottom left: Reaction rates for thiamine standards. Right: Plots of fluorescence intensity versus time for conversion of thiamine to thiachrome for four thiamine standards. Linearity indicates a constant reaction rate for each standard.

Fluorescence kinetics are often measured using a fast-mixing accessory called a stopped flow. The stopped flow mixes two or more solutions together on the order of a few milliseconds so that the binding or reaction can be recorded closer to mixing time zero, without the effects of diffusion.

Here is shown the binding of a fluorophore called ANS to a protein, BSA. ANS fluorescence increases upon binding, so the rate of binding can be measured using fluorescence kinetics. In this example, the binding of ANS to BSA occurs with a rate of approximately 400 milliseconds.

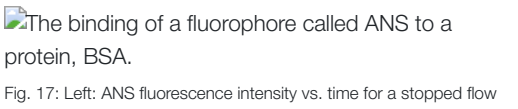
The binding of a fluorophore called ANS to a protein, BSA.

Fig. 17: Left: ANS fluorescence intensity vs. time for a stopped flow mixing of BSA and ANS to measure ANS binding to the protein measured on a HORIBA FluoroMax-4. Top Right: Stopped flow fast mixing accessory. Bottom right: Schematic of the stopped flow accessory

How can I control the sample temperature on a fluorometer?

Circulating baths and Peltier temperature controllers are two accessories that will control the temperature of a sample on a fluorometer. “Standard” cuvette holders on fluorometer systems have two connections to allow for liquid circulation, which can be connected to a recirculating water bath. This enables temperatures in the range -40°C to 70°C to be regulated.

An alternative method is to use a Peltier-controlled cell holder, which has a faster response than a water bath, and enables temperatures in the range -25°C to 105°C to be regulated. The difference is that a Peltier device will control the temperature much more precisely than a circulating batch, which tends to ramp to a temperature, overshoot it and come back until the temperature is reached.

Circulating baths are good if a temperature needs to be set and held during an experiment, but for measuring samples at different temperatures over a range, or for samples that are very sensitive to temperature changes, a Peltier temperature controller is a more practical choice. It is also possible to use cryostats and mounting kits which are available for different models of liquid nitrogen and helium cryostats. Besides cooling, most of the cryostats can also heat samples to 500K and higher.

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