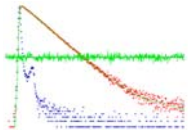


Time-resolved fluorescence anisotropy

Fluorescence anisotropy measures the depolarisation of the fluorescence emission. The main reasons for depolarisation include the energy transfer to another molecule with a different orientation or molecular rotation caused by Brownian motion. Molecular motion depends on local environmental factors, such as viscosity and molecular confinement, and the size of the molecule. Thus a measurement of fluorescence anisotropy is useful in obtaining information concerning molecular size and mobility.

Molecular rotation

Most fluorophores absorb light in a preferred direction (parallel to their absorption dipole). If polarised light is used to excite a sample, then only a subset of molecules, whose absorption dipoles are parallel to that light, will be excited. The excited molecules are not static and Brownian motion causes this subset to become disordered. With sufficient time the fluorescence emission will result from randomly oriented molecules. By monitoring both parallel and perpendicular planes of polarisation it is possible to follow this path from order to disorder, as illustrated in Fig. 1.

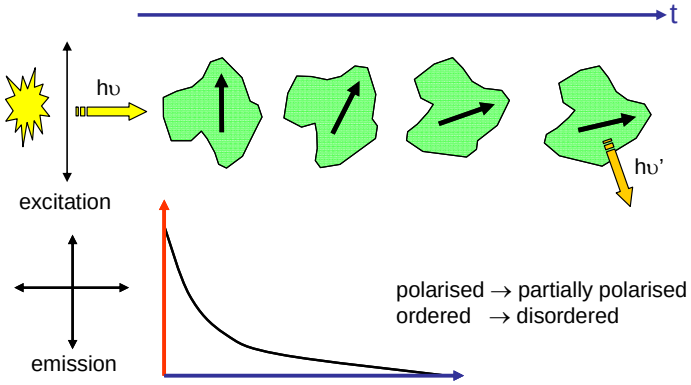


Fig. 1. Schematic representation for molecular rotation after absorption of vertically polarised light, which is emitted from a random orientation (assumes absorption and emission dipoles parallel). The change in anisotropy (red axis) with time (blue axis) is indicated.

A measure of this is the fluorescence anisotropy, *r*, which relates to the intensities (*I*) of the planes (↕,↔) of polarisation, defined as,

$$r = \frac{I_{\updownarrow} - I_{\leftrightarrow}}{I_{\updownarrow} + 2I_{\leftrightarrow}}$$

In the simplest case the change of anisotropy with time is given by,

$$r(t) = r_0 \exp(-t/\tau_r)$$

Where r_0 is the initial anisotropy and ranges from 0.4 (parallel transition dipoles) to -0.2 (perpendicular dipoles). Note that, these values are different if using 2-photon excitation. τ_r is the rotational correlation time, which can be considered a measure of the order-disorder process.

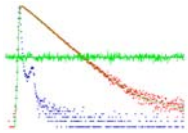
The steady state anisotropy can be represented by,

$$\bar{r} = r_0 \sum_i \left[\frac{\alpha_i}{1 + t/\tau_{ri}} \right]$$

Although simpler to measure, this gives an incomplete description (only gives $\bar{r}$) of the process compared to a time resolved measurement, which enables r_0 and τ_r, as well as the fluorescence lifetime, to be determined. The rotational correlation time can be related to the rotational diffusion coefficient (D_r) and in the simplest case to the effective volume (V) and local viscosity (η) by the following,

$$\tau_r = \frac{1}{6D_r} = \frac{V\eta}{kT}$$

Here k is Boltzman's constant and T the absolute temperature. Thus, a time-resolved measurement returning a value of τ_r can be used to provide information concerning molecular size and the fluidity of the medium in which the molecule is situated. Although, care in interpretation is required as only equivalent viscosities and relative changes can be realistically determined.



Hindered rotation

If the fluorophore is not fully free to rotate, then a non zero limiting anisotropy (r_∞), manifest in the anisotropy decay below (Fig. 2.), can be considered.

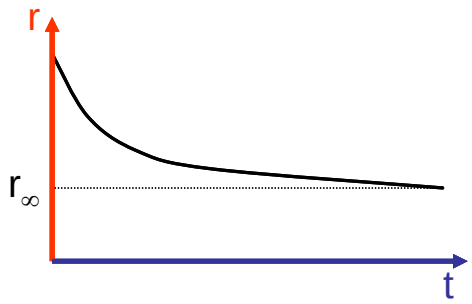


Fig. 2. Illustration of a limiting value (r_∞) in the anisotropy decay

The time-resolved measurement is then described as follows,

$$r(t) = r_\infty + (r_0 - r_\infty)\exp(-t/\tau_r)$$

Considering a "wobble in cone" model, the ratio of the initial and limiting anisotropies can be used to calculate a semicone angle, which reflects the degree of orientational constraint exercised by the medium in which the molecule is situated.

Time-resolved measurements

There are some practical considerations that need to be taken into account when measuring time-resolved anisotropy. Some aspects are briefly considered below;

- **Choice of probe molecule**

The lifetime of the probe should be similar to τ_r. If the lifetime is much shorter than τ_r, the fluorescence is over before the molecular rotation is complete, making determination of τ_r problematic.

- **Equipment polarisation bias**

Detectors and monochromators may have a bias for one plane of polarisation over another. A correction (g-factor) measurement should be done, involving the use of horizontally polarised excitation incident on the sample. Note that the g-factor is wavelength dependent, so needs repeating if different measurement conditions are used.

- **Fit to difference or raw anisotropy data**

The raw anisotropy data may contain instrumental distortion, so fitting to the difference file (obtained from data analysis and relates to the numerator in the first equation) is advisable. Using reconvolution removes this distortion and enables short rotational correlation times to be recovered.

Additional use of polarised measurements

It should be noted that when exciting a sample with a fast polarised laser pulse, polarisation effects can be present. This can make the regular fluorescence decay appear more complex and can relate to depolarisation effects. It is advisable to use a vertically orientated polarizer on the excitation and the emission polarizer at the magic angle (54.7° to the vertical) to remove these depolarisation effects.

Applications

- uncovering homoFRET
- molecular interactions / binding
- energy migration
- local viscosity
- molecular confinement
- membrane phase transition