Anisotropy Measurement Using the Agilent Cary Eclipse Fluorescence Spectrophotometer

A Versatile Instrument for Accurate Measurements

Technical Overview

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Introduction

A scientific measurement is only useful if it accurately records the characteristics of the sample. To ensure that each measurement is representative, the careful analyst must usually check that sample concentration or volume does not change during sample analysis, that there is no deviation from the expected relationship between analyte concentration and signal intensity, and that no physical change or photodegradation of the sample takes place. However, none of these complicating factors are of concern for the analyst who uses an Agilent Cary Eclipse Fluorescence spectrophotometer to undertake fluorescence anisotropy measurements. This is because fluorescence anisotropy is a nondestructive yet powerful analytical technique that provides robust and reliable measurements that are free from the influence of external variables. The Cary Eclipse Fluorescence spectrophotometer is the only instrument that can reliably measure samples and not cause photodegradation. Because of the intrinsic analytical robustness of fluorescence anisotropy, it is now routinely used to yield accurate, precise, and repeatable data in several key application areas (Table 1). Fluorescence anisotropy measurements have led to a heightened degree of accuracy, significant scientific advances, and important application developments. The advantage of fluorescence measurements made using the Cary Eclipse is that all of this can be accomplished without damaging your precious samples.

Technique summary

Fluorescence anisotropy measurements are made using waves of light that are all aligned in a single direction (polarized). When light hits a sample, it interacts with the molecules and can generate a secondary signal that can be measured. If polarized light is used to analyze the sample and molecules in the sample are free to move or rotate, not all of this secondary signal will be polarized. In anisotropy, we accurately measure how highly polarized the emitted light is after it has interacted with the sample. This can be a powerful probe into the characteristics of a sample. It also means that anisotropy measurements can provide detailed information about the size and shape of molecules, how easily they can move, and how they change during chemical processes or through variations of time or temperature.

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A polarizer is an optical device that allows light of only one orientation to pass through, while rejecting light of all other orientations. In the Cary Eclipse Fluorescence spectrophotometer, polarizers can be placed in the beam before and after the sample. Agilent Cary WinFLR software is then used to record the results of each polarized measurement, and to swap between different types of polarization measurement (anisotropy, polarization, or sum function). The Cary WinFLR software automatically shows the sample spectra and, if appropriate, the result of the anisotropy calculation. Cary WinFLR software can also be used to automatically guide the user through each anisotropy measurement by providing real-time, step-by-step tuition as the experiment progresses.

Table 1. Some Key Application Areas for Fluorescence Anisotropy

Area	Application
Polymer science	Understand polymer viscosity
	Identify molecular orientation
	Characterize phase transition temperature and type
	Local microviscosity in liquids and polymers
Biological systems	**Monitoring FRET between identical molecules (homo-FRET)**
	Investigate enzyme reactions
	Determine molecular alignment
	Estimate the internal viscosity of membranes
	Investigate membrane fluidity and permeability
Molecular biology	**Size and denaturation of proteins**
	Real-time study of protein-protein interactions
	Study of protein crowding and oligomerization
	Measure protein dynamics
	Characterize DNA-protein interactions
	Investigate DNA/RNA hybridization
	Study DNA/RNA structures
	Examine myosin reorientation
	Ligand affinity screening
	Degradative assays
Immunology	**Antigen-antibody interaction**
	Immunoassay studies
	Receptor-ligand binding
Academia	Understand fundamental physical properties of species
	Investigate solvent relaxation effects
	Chain dynamics
	Multicomponent determination
	Molecular aggregation
Materials	**Quantify the size of nanoparticles (quantum dots) and macromolecules**
	Energy transfer in optoelectronic materials
	Investigate guest-host systems (micelles, porous materials, metal-organic frameworks)
	Anisotropic properties of metal-ligand complexes

Samples in applications highlighted in bold text may be photosensitive, and can be readily and irreparably altered when exposed to light from many fluorescence spectrophotometers. The lamp in the Agilent Cary Eclipse Fluorescence spectrophotometer only flashes to acquire a data point. This key technological advantage prevents photodegradation and ensures that these critical samples can be measured without concern [1].

Interpreting anisotropy data

The fluorescence anisotropy of a sample is largely controlled by how easily the constituent molecules can move around and change orientation. If all the light that first interacts with a sample is polarized in a single direction, but the Cary Eclipse Fluorescence spectrophotometer records that the emitted light is less polarized, then we can learn a great deal about that sample. Generally, we know that the more viscous a sample, the more rigidly the molecules are held in position. We also know that larger molecules rotate slower than smaller ones. These general principles form the basis of the anisotropy measurements that are routinely used in countless life science applications, to monitor how molecules behave in different key substances, or as a function of, for example, temperature or time.

Example application: a well-characterized molecule dissolved in three different media

Molecules in a thermoplastic polymethylmethacrylate (PMMA) block are held so tightly that they are essentially frozen. The molecules have a limited ability to move and so yield a high anisotropy value (Figure 1). In a highly viscous liquid such as ethylene glycol they are less strongly held, and in highly fluid media such as water they are largely free to move.

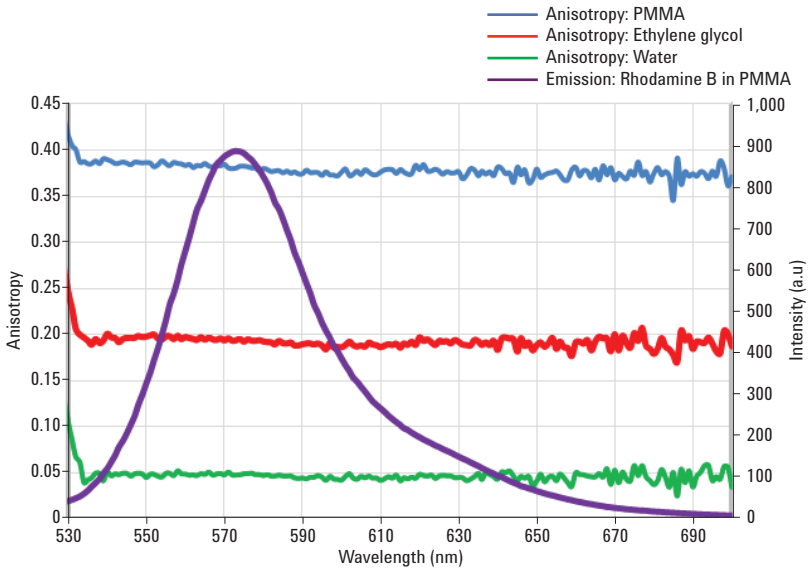

Figure 1. The results of anisotropy measurements of rhodamine B in water (green line), ethylene glycol (red line), and thermoplastic (PMMA) block (blue line) illustrate that large differences in the viscosity of each medium are reflected by large differences in the calculated anisotropy. In water, which does not inhibit the movement of molecules, the anisotropy value is close to zero, whereas the PMMA block is exceptionally good at holding molecules extremely tightly; so tightly, in fact, that the high anisotropy value calculated for rhodamine B in the PMMA sample approaches the theoretical maximum [2]. The anisotropy of rhodamine B in ethylene glycol sits comfortably between the values calculated for water and the PMMA block. This reflects the higher viscosity of ethylene glycol compared to water but greater fluidity relative to the PMMA block. The purple line shows an emission spectrum for rhodamine B in PMMA as collected with vertically polarized light and with a vertical polarizer in the emission beam using the Agilent Cary Eclipse Fluorescence spectrophotometer.

Example application: accurately characterize molecular structures

It can be difficult to quantitatively determine the amount of a compound in any experiment that follows a temperature profile. This is because sample signals may be as affected by temperature variations as a genuine change in sample concentration. Fluorescence anisotropy is a reliable technique for removing the complicating effects of the physical environment because it only yields information about the molecular sample of interest. Therefore, the effects of temperature are automatically isolated from variations in sample chemistry (Figure 2).

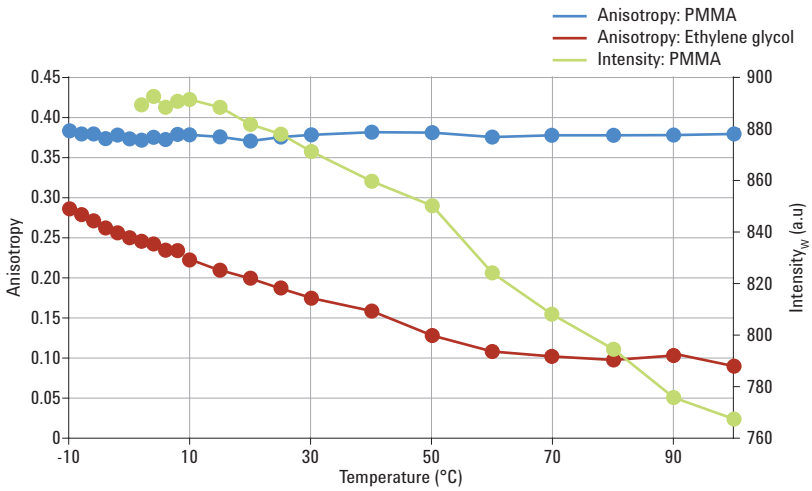

Figure 2. The results of anisotropy measurements of rhodamine B dissolved in ethylene glycol and a thermoplastic (PMMA) block at different temperatures. The green line shows the effect of temperature on the absolute intensity of the rhodamine B signal as a function of temperature. This signal is the result of a complex interaction between different factors associated with the behavior of molecules as temperature changes and, if interpreted incorrectly, may lead the unwary to believe that the concentration of rhodamine B decreased as temperature increased or, perhaps, that the structure of rhodamine B altered significantly with temperature. However, the blue line shows the results of anisotropy measurements made across an identical temperature range. It shows that the fluorescence intensity does not affect the anisotropy, and that the structure of the PMMA block is not temperature-dependent. The data show that the thermoplastic block is exceptionally good at holding rhodamine B molecules tightly from –10 to 100 °C. The same cannot be said for ethylene glycol. The results of the anisotropy experiments (red line) show that the structure of ethylene glycol changes as temperature changes, and that, as temperature increases, this allows the rhodamine B molecules more freedom to move around (the anisotropy decreases).

Advantages of the Agilent Cary Eclipse Fluorescence spectrophotometer: accurate anisotropy measurements of photosensitive samples

You just have to look at any product that has been exposed to sunlight for a while to know that light can easily degrade many organic and inorganic compounds. This common phenomenon can also be a huge concern in the laboratory where the fluorescence spectrophotometer might be progressively degrading your samples during every measurement. More critical still, many of the species that benefit most from the increased sensitivity offered by fluorescence anisotropy measurements (Table 1) are susceptible to photobleaching. In essence, structural and compositional changes to your sample caused by interaction with the light beam will bring the accuracy of the analyses into question. This is potentially frustrating because the integrity of your data is at risk from the very instrument you use to characterize your samples, and doubly so because it can easily be prevented. With its unique optical configuration, the Cary Eclipse Fluorescence spectrophotometer gives you huge analytical advantages. The source lamp in the Eclipse is only on when it is acquiring a data point (it flashes 80 times every second), and because it is switched off completely between each flash, energy from the lamp that can cause photochemical damage does not interact with the sample. This high-speed process has been proven to eliminate photodegradation [1] and ensures that true and meaningful data can be collected from various critical chemical and biological, light-sensitive samples without compromise to accuracy and reproducibility.

Conclusions

The examples in this study demonstrate that fluorescence anisotropy measurements are a powerful tool in the collection of important molecular information from a range of sample types. Fluorescence anisotropy allows the molecular environment of materials to be accurately imaged and analyzed quickly and without the interfering influences that plague many similar analytical techniques. We have shown that the Agilent Cary Eclipse Fluorescence spectrophotometer can easily be used to illustrate differences in the molecular properties of three common media.

References

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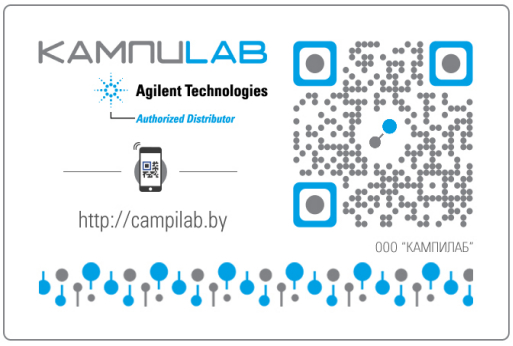

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