Imaging is NOT just a black and white issue.

Application Notes: Fluorescence Polarization

INTRODUCTION

While the majority of fluorescence microscopists investigate cell structure and function using only the intensity information from spectral emission, an increasing number of scientists are turning their attention to the technique of fluorescence polarization to investigate different biochemical properties of fluorescent samples. This technique is being used to probe different intrinsic and extrinsic causes of depolarization, which can subsequently be used to infer physical properties. An example of an intrinsic cause of depolarization would be an angular displacement between the absorption and emission dipoles of a fluorophore. An extrinsic cause is one that acts during the lifetime of the excited state.[1] These include rotational diffusion of the fluorophore and radiationless energy transfer among fluorophores (e.g. FRET). When these effects occur on the order of the time scale of the rate of fluorescent emission, the depolarization can be significant.

Polarization and anisotropy measurements are often used to estimate the rate and extent of rotational diffusion during the lifetime of the excited state. These, in turn, have been used to quantify different biochemical properties such as protein denaturation, protein-ligand association reactions, and the rotational rates of proteins. The anisotropies of fluorophores bound to membranes have been measured to estimate the internal viscosities of membranes and the dependency of membrane phase transitions on membrane composition.[2]

EXAMPLE APPLICATION: ANISOTROPY/FLIM

Recent research has shown that the Dual-View™ system can be used to simultaneously acquire 2-D polarization and lifetime images, which can then be transformed to yield maps of rotational correlation time and fluorescence lifetime.[3] This technique has been labeled time-resolved fluorescence anisotropy imaging (TR-FAIM), which is an extension of time-domain FLIM. It utilizes linearly polarized laser pulses to excite a sample. The parallel and perpendicular components of the fluorescence emission of the sample are then imaged simultaneously using the Dual-View™. Figure 1 shows the result of applying this technique to the images several wells of a standard multiwell plate with rhodamine 6G in methanol, ethylene glycol, trimethylene glycol and glycerol. The acquired images are processed to recover the rotational correlation times and unperturbed fluorescence lifetimes of the sample. These, in turn, are used to determine the local viscosity and refractive index of the probe environment.

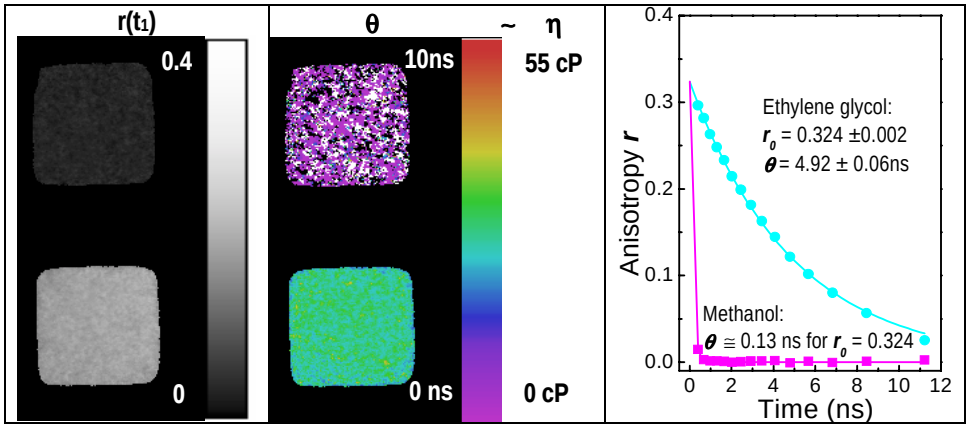

Figure. 1: TR-FAIM results of 2 wells filled with rhodamine 6G in methanol (top) and ethylene glycol (bottom). (Left Panel) Transient anisotropy 400 ps after the excitation pulse, showing strong anisotropy in ethylene glycol and apparently weak anisotropy in the sample in methanol since it has already decayed almost entirely. (Middle Panel) Map of the rotational correlation time θ in a false colour scale from 0 ns to 10 ns. The time scale has also been converted directly into a viscosity scale ranging from 0 cP to 55 cP. (Right Panel) The anisotropy decays averaged over the individual well areas of (Middle Panel). Images courtesy of Siegel et. al.

[1] Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, Chapter 5.
[2] Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, Chapter 5.
[3] Siegel, J., Suhling, K., Leveque-Fort, S., Webb, S.E.D., Davis, D.M., Phillips, D., Sabharwal, Y., French, P.M.W., "Wide-field time-resolved fluorescence anisotropy imaging (TR-FAIM) – A novel approach to image the viscosity of a fluorophore environment," Accepted for publication in Review of Scientific Instruments.

SYSTEM CONFIGURATION

Optical Insights' Dual-View™ has been used with tremendous success in various fluorescence imaging applications for microscopy. In most applications, the Dual-View™ is fitted with dichroic filters and spectral emission filters for simultaneous imaging of multiple fluorescent labels. However, the Dual-View™ can also be fitted with a polarization beamsplitter and two optional polarization filters for polarization imaging of fluorescent samples. This configuration allows for the simultaneous acquisition of two polarization images, one parallel to the polarization of the source and the other perpendicular to the polarization of the source. These images are, in turn, used to calculate the two-dimensional polarization/anisotropy of the sample. The operation of this system is shown conceptually in Figure 2.

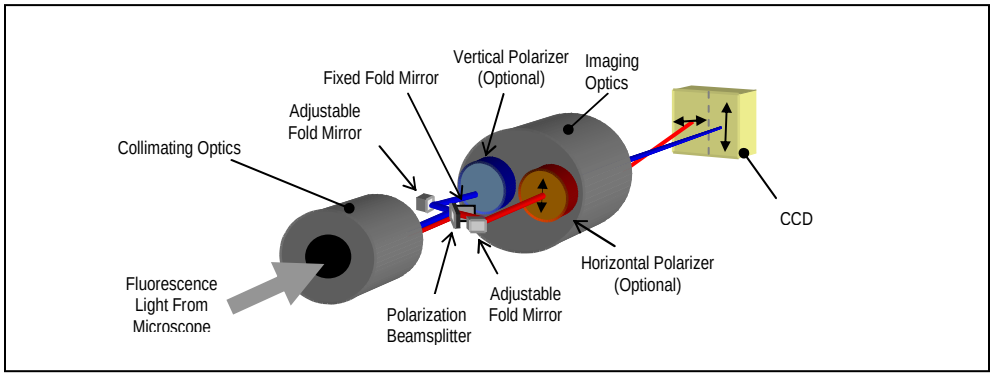

Figure 2: Operation of Dual-View™ fluorescence polarization imaging system.

TECHNIQUE SUMMARY

For anisotropy measurements, a fluorescent sample is excited with polarized light yielding fluorescent emission from the sample. This simple property of fluorescent samples can be used to probe different biochemical properties of cellular structures. When implemented on a microscope, this technique requires excitation of the sample with vertically polarized light. On the emission side, two separate images need to be acquired. One image is taken with a vertically polarized filter in one emission channel and the second image is taken with a horizontally polarized filter in the other emission channel. The first image is $I_{\parallel}$ and the second image is $I_{\perp}$. These acquired images can then be used to determine the polarization (P) and anisotropy (r) at each pixel in the image. The mathematical relationship is shown with the equations below.

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad \text{and} \quad r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad \text{or alternatively,} \qquad P = \frac{(I_{\parallel}/I_{\perp}) - 1}{(I_{\parallel}/I_{\perp}) + 1} \quad \text{and} \quad r = \frac{(I_{\parallel}/I_{\perp}) - 1}{(I_{\parallel}/I_{\perp}) + 2}$$

If both images have been acquired simultaneously, using the ratio of the images has significant advantages because it normalizes for fluctuations in source intensity, photobleaching, and uneven dye loading.

Note: Before performing these calculations, it is important that you have properly calibrated all the instrumentation being used for your experiments. If you have any questions about calibration procedures, please contact Optical Insights, LLC.

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