Wide-Field Time-Resolved Fluorescence Anisotropy Imaging (TR-FAIM)


Summary

Polarization-resolved fluorescence lifetime imaging (FLIM) can provide information about the rotational diffusion of a fluorophore. We have developed a wide-field time-resolved fluorescence anisotropy imaging (TR-FAIM) system to quantitatively image both the fluorescence lifetime and the rotational correlation time of a fluorophore. Upon excitation with linearly polarized light, we simultaneously image the parallel and perpendicular polarization components of the fluorescence emission employing a polarization-resolved imager in front of a time-gated intensified CCD. We demonstrate this technique by imaging the rotational correlation time of fluorescein in solutions of varying viscosity in a multiwell plate and in live cells.

Introduction

Fluorescence imaging is an important technique in the biological sciences, since it can be performed on live cells and tissue. The fluorescence emission of dyes or proteins can be characterised not only by intensity and location, but also by fluorescence lifetime, wavelength and polarization. Fluorescence lifetime imaging (FLIM) [1], for example, provides contrast according to the fluorescence lifetime which is the average time a fluorophore remains in its excited state after excitation. While the fluorescence lifetime depends on the intrinsic characteristics of the fluorophore itself, it also depends on its local environment. For example, we have recently shown that the fluorescence lifetime of the green fluorescent protein is a function of the refractive index of its microenvironment [2]. Interactions with other molecules, e.g. by fluorescence resonance energy transfer (FRET), can also affect the fluorescence lifetime. Thus, FLIM allows the microenvironment of a fluorophore to be imaged.

Polarization-resolved FLIM allows complementary information about the fluorophore’s environment to be obtained. When excited with linearly polarized light, rotational diffusion of the fluorophore in its excited state results in a depolarization of the fluorescence emission, which depends on the viscosity and the size and shape of the fluorophore. Therefore, the viscosity of the fluorophore’s environment, or binding of the fluorophore to other molecules can be examined with this method. Recently, polarization-resolved frequency-domain by FLIM imaging polarization components sequentially was reported [3]. We subsequently reported the extension of time-gated FLIM to time-resolved fluorescence anisotropy imaging (TR-FAIM) by simultaneously imaging the parallel and perpendicular polarization components of the fluorescence emission [4]. Acquiring the polarization components simultaneously reduces artefacts that could be caused by instrumental drift, photochemical changes or movement, which may be experienced when acquiring sequentially, and it is also faster. Fig. 1 schematically shows the experimental set-up using a polarization-resolved imager (PRI, Optical Insights, www.optical-insights.com) in conjunction with our wide-field FLIM system [4]. We have now introduced a background subtraction to permit quantitative viscosity imaging in multiwell plates and living cells [5].

Theoretical Background

The time-resolved fluorescence anisotropy r(t) can be defined as

\[ r(t) = \frac{I(t) - B - G(I(t) - B)}{I(t) - B + 2(GI(t) - B)} \tag{1} \]

Fig. 1.(a): Experimental set-up of the wide-field TR-FAIM instrument (polarization-resolved time-gated FLIM). The Polarization-Resolved Imager (PRI) contains a polarizing beamsplitter and adjustable mirrors. Using a C-mount adapter, the PRI is mounted onto the gated optical image intensifier (GOI), the output phosphor screen of which is imaged with a CCD camera. The PRI splits a single image in image plane 1 into two spatially identical images differing only by their polarization (image plane 2), which are thus recorded simultaneously. (b) A series of such polarization-resolved fluorescence intensity image pairs are acquired at various delays after the excitation pulse to sample their fluorescence decay profiles [4].

K. Suhling

where $I_p(t)$ and $I_\perp(t)$ are the fluorescence intensity decays parallel and perpendicular to the polarization of the exciting light. G accounts for different transmission and detection efficiencies of the imaging system at parallel and perpendicular polarization, and B accounts for a non-zero background [5]. For a spherical rotor, r(t) decays as a single exponential:

$$r(t) = (r_0 - r_\infty) \exp \left( -\frac{t}{\theta} \right) + r_\infty$$

(2)

where $\theta$ is the rotational correlation time, $r_0$ is the initial anisotropy and $r_\infty$ is the limiting anisotropy which accounts for a restricted rotational mobility. For a spherical rotor in an isotropic medium, $\theta$ is directly proportional to the viscosity of the solvent and the volume V of the rotating molecule:

$$\theta = \frac{\eta V}{kT}$$

(3)
where \( k \) is the Boltzmann constant and \( T \) the absolute temperature.

**Results**

**Multiwell Plates**

To demonstrate quantitative imaging of the solvent viscosity, we placed fluorescein in mixtures of glycerol and NaOH solutions in a multiwell plate [5]. Fig. 2(a) shows how the glycerol percentage (by weight) varied across the field of view. The samples were excited at 470nm using 100fs laser pulses and a series of polarization-resolved time-gated fluorescence images were recorded, as shown schematically in Fig. 1(b). Fluorescence anisotropy images were calculated according to equation (1) with an appropriate background value and fitted to a single exponential (equation (2)) with the offset fixed to zero \( r_\infty = 0 \) [5]. Fig. 2(b) shows an image of the rotational correlation times with clearly discernible contrast over more than one order of magnitude across the sample array. Plotting the rotational correlation time versus the solvent viscosity yields a linear plot (Fig. 2(c)). Assuming a spherical molecule, a hydrodynamic fluorescein radius of 0.54nm can be calculated from the gradient of a straight-line fit through zero according to equation (3). This value, derived from the image in Fig. 2(b), is in good agreement with values derived from other, non-imaging single-point studies and demonstrates the quantitative accuracy that can be achieved with this imaging method [5].

**Biological Samples**

Using an inverted wide-field microscope, TR-FAIM was applied to living B cells stained with the fluorescein derivative carboxyfluorescein diacetate succinimidyl ester (CFSE), which can bind to intracellular proteins (Molecular Probes, www.probes.com). The rotational correlation time is expected to report on the viscosity of the cytoplasm, and the corresponding image is shown in Fig. 3(a), with the averaged fluorescence anisotropy decay curve in Fig. 3(b). The correlation time averaged over the entire cell is around 1.5ns at 20°C, which, assuming an effective hydrodynamic radius of 0.62nm for CFSE, corresponds to an average cytoplasmic viscosity of 6.0cp. This value lies within the range expected in these conditions. In addition, we obtain a limiting anisotropy \( r_\infty = 0.08 \), which can be interpreted in terms of a hindered rotation of the probe due to binding in the cytoplasm [5].

**Conclusions**

These results illustrate the potential of time-resolved fluorescence anisotropy imaging to image the viscosity in multiwell plates and in living cells. HomofRET, which occurs at close proximity of two identical fluorophores and causes a depolarization of the emitted fluorescence, can also be identified with this technique [3]. Moreover, as the rotational diffusion can be slowed down by binding, TR-FAIM has potential to visualise the binding of ligands and receptors in the cell. The approach presented here can be extended in order to overcome the limited depth discrimination in wide-field microscopy by combining it with wide-field optical sectioning techniques such as structured illumination, multibeam multiphoton microscopy or tandem scanning confocal microscopy.

**References**


