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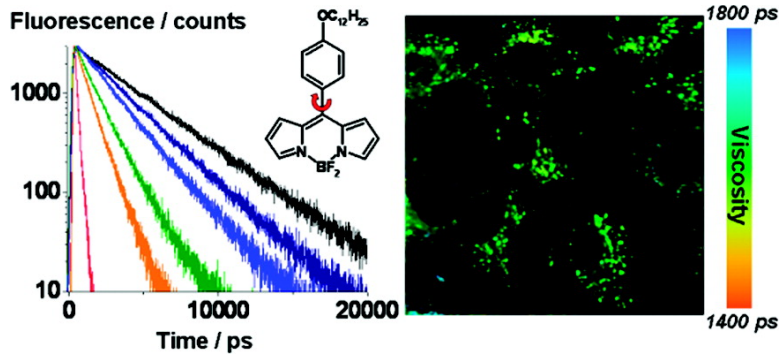


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# Molecular Rotor Measures Viscosity of Live Cells via Fluorescence Lifetime Imaging

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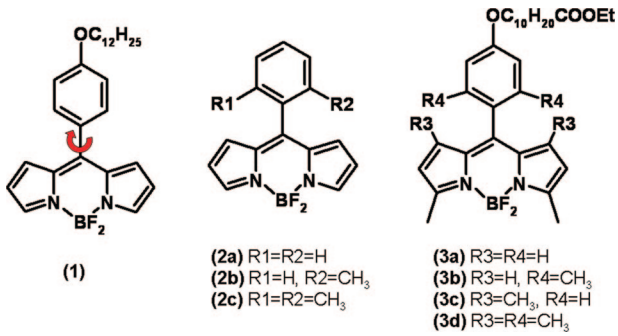
Viscosity is one of the major parameters determining the diffusion rate of species in condensed media. In biosystems, changes in viscosity have been linked to disease and malfunction at the cellular level.[1] These perturbations are caused by changes in mobility of chemicals within the cell, influencing fundamental processes such as signaling and transport and the efficiency of bimolecular processes governed by diffusion of short-lived intermediates, such as the diffusion of reactive oxygen species during an oxidative stress attack. While methods to measure the bulk macroscopic viscosity are well developed, imaging local microscopic viscosity remains a challenge, and viscosity maps of microscopic objects, such as single cells, are actively sought after.[2–6] We report a new approach to image local microviscosity using the fluorescence lifetime of a molecular rotor.

Molecular rotors, in which the nonradiative decay of the fluorescent excited state is influenced by the viscosity of the medium, have been recently highlighted as promising candidates for measurements of local viscosity using the change of fluorescence quantum yield.[6] However, fluorescence intensity-based measurements suffer from uncertainties in the calibration of rotor responses. It can, for example, be altered by the fluctuations in the fluorophore concentration and inhomogeneities in the optical properties of the medium as well as by viscosity. A ratiometric approach, using probes that incorporate two independent chromophores, has been suggested to overcome this problem, when one of the chromophores is not influenced by viscosity and is used to gauge the concentration while the other acts as a molecular rotor.[2,7] Our present approach based on fluorescence lifetime determination does not require conjugation of the molecular rotor to another fluorescence label. It represents a major advance due to ease of system calibration, ultrasensitive detection, reduced complexity of the optical setup compared to ratiometric approach, and the ability to analyze spatially resolved fluorescence decays using fluorescence lifetime imaging (FLIM).[8]




We hypothesized that *meso*-substituted 4,4′-difluoro-4-bora-3a,4a-diaza-*s*-indacene (**1**) can act as a molecular rotor based on the previous literature data for similar compounds. For example, for **2a**, it was reported that the fluorescence intensity decreases with increasing temperature when the dark nonemissive state becomes accessible.[9] Furthermore, a DFT study of **2a** has confirmed that the dark state is accessed by rotating the phenyl group from the metastable twisted conformation (corresponding to an emissive state) into the plane of the dipyrrin framework.[9] In **2b** and **2c**, where the free rotation of the phenyl group is restricted, nonradiative decay is prevented, and this leads to an increase in the fluorescent quantum yield which becomes very close to unity.[10] Similarly, in the series of **3a**−**d**, the fluorescence quantum yield increases from **3a** < **3b** ≈ **3c** < **3d**, following the decreasing ease of rotation of the *meso*-phenyl group.[11]

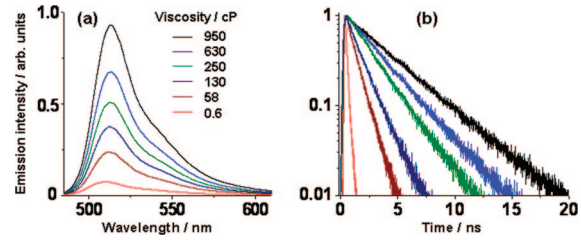



***Figure 1.*** Fluorescence spectra (a) and decay traces (b) recorded for **1** in methanol/glycerol mixtures of different viscosity.

Fluorescence measurements of **1** made in methanol/glycerol mixtures of different viscosities (Figure 1a) show that the fluorescence quantum yield increases dramatically with increasing solvent viscosity. The observed increase in fluorescence intensity is consistent with the restricted rotation of the phenyl group in the medium of high viscosity, thus preventing relaxation via the populating of the dark excited state. In addition, the fluorescence lifetime also increases from 0.7 ± 0.05 to 3.8 ± 0.1 ns with increasing solution viscosity from 28 to 950 cP (Figure 1b). The rates of radiative and nonradiative decays were calculated from the measured fluorescence lifetimes and quantum yields and are presented in Figure S2 in the Supporting Information. From these results, we conclude that, for **1**, the nonradiative decay rate increases with decreasing viscosity, and the radiative decay rate remains approximately constant. Thus **1** is truly a molecular rotor which displays both fluorescence intensity and lifetime sensitivity to viscosity of the environment. The measured fluorescence lifetimes are >100 ps, falling in the range accessible with time correlated single photon counting (TCSPC) offering ultrasensitive and precise fluorescence lifetime detection.[12]

The calibration plot of fluorescence lifetime $\tau$ versus viscosity $\eta$ for **1** is shown in Figure 2a, and it is described well by eq 1:[13]

$$\tau = zk_0\eta^{\alpha} \quad (1)$$

where $k_0$ is the radiative rate constant, and $z$ and $\alpha$ are constants.

The plot of log $\tau$ versus log $\eta$ (Figure 2a, inset) is fitted by a straight line with a slope of 0.50 ± 0.03, in agreement with the literature data for molecular rotors in viscous media.[13,14]

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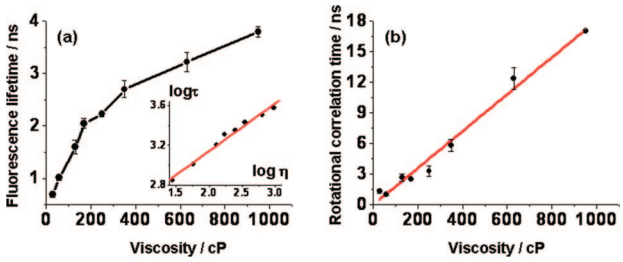


***Figure 2.*** The changes of fluorescence lifetime (a) and rotational correlation time (b) for **1** as a function of viscosity.

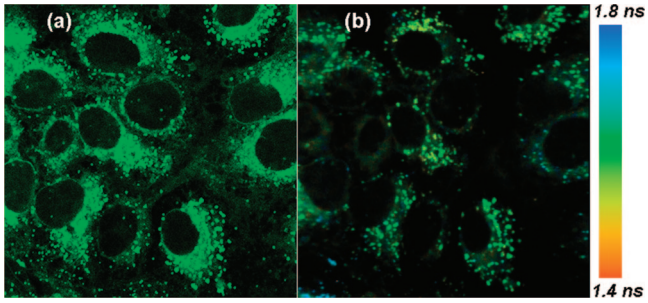


***Figure 3.*** (a) Confocal fluorescence image (114 × 114 $\mu$m) obtained following 488 nm excitation, 550 ± 25 nm fluorescence detection from SK-OV-3 cells incubated with 1 $\mu$M solution of **1**; (b) FLIM image obtained following 467 nm pulsed excitation of the same layer of cells.

In the medium of high viscosity, the rotational Brownian motion of the molecule as a whole is slowed down. The rotational diffusion rate can be determined by time-resolved fluorescence anisotropy using polarization-resolved TCSPC. We have recorded time-resolved fluorescence anisotropy decays following an increase in viscosity from 28 to 950 cP. The rotational correlation time $\theta$ of **1** increases linearly with solvent viscosity (Figure 2b). The slope of this plot allows us to estimate the dimensions of the rotating unit, according to the Stokes−Einstein relation:

$$\theta = \eta V/kT \qquad (2)$$

Fitting the experimental data to this equation yields an apparent value of 74 Å$^3$ for the volume $V$ of the sphere and ca. 3 Å for the radius of the rotor. The intercept is 0.08 ± 0.4 ns, in agreement with the zero intercept predicted by eq 2. Thus the time-resolved fluorescence anisotropy of **1** can be used together with the fluorescence lifetime to map the viscosity.[3−5]

We have incubated **1** in cells and used FLIM to determine the viscosity. The confocal fluorescence image of cells incubated with **1** is shown in Figure 3a and clearly shows the intracellular uptake of the molecular rotor with punctate dye distribution, probably in the endocytotic vesicles (see Supporting Information). The FLIM image obtained using excitation with a pulsed diode laser at 467 nm shows a narrow lifetime distribution between 1.4 and 1.8 ns (Figure 3b and Figure S4 in the Supporting Information). According to our calibration graph, this range of lifetimes of **1** inside the cell corresponds to an average viscosity of 140 ± 40 cP. The fluorescence lifetime map of molecular rotor **1** within live cells provides the direct measurement of intracellular viscosity with the spatial resolution of a confocal microscope, giving a level of detail unavailable previously.

To ensure that this high viscosity value does not result from the binding of the rotor to the intracellular targets, which could restrict the rotation of the phenyl group, we have performed time-resolved fluorescence anisotropy measurements of the cells. The average value of $\theta$ obtained from cells is 1.1 ns (Figure S5 in Supporting Information), corresponding to an intracellular viscosity of ca. 80 cP, according to the anisotropy calibration graph (Figure 2b).[15] The average viscosity value 80 cP is in the same order of magnitude of that given by FLIM. This fast rate of anisotropy decay indicates that the rotation of **1** is not restricted by binding to intracellular targets since the calibration graph for $\theta$ corresponds to the rotor size of ca. 3 Å, and this value will significantly increase upon binding.

The anisotropy and FLIM measurements indicate that **1** inside SK-OV-3 cells locates in an environment that is more viscous than water (1 cP) and cellular cytoplasm (1−2 cP)[2−5] by approximately 2 orders of magnitude. The location of the rotor is probably determined by its hydrophobicity, which is further increased by the incorporation of $C_{12}$ chain. The high viscosity of the hydrophobic cellular organelles was suggested previously[16] and could be the crucial factor increasing the efficiency of processes involving reactive oxygen species by increasing their lifetime, for example, that of cytotoxic singlet oxygen.[17]

In summary, we have developed a practical and versatile approach to measuring the microviscosity of the environment of a molecular rotor, based on the determination of its fluorescence lifetime. This method is applicable to biological systems and is unique in providing both spatial resolution and precision of viscosity determination. The variety of fluorescent molecular rotors can be developed to report on specific cell targets.

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**Supporting Information Available:** Experimental details, additional spectroscopic and imaging data. This material is available free of charge via the Internet at http://pubs.acs.org.

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