Supporting Information

Molecular Rotor Measures Viscosity of Live Cells via Fluorescence Lifetime Imaging

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(1) Experimental procedures
Synthesis and characterization of 1.

Compound **1** was synthesized by the method of Wagner and Lindsey.[1]
DDQ (280 mg, 1.23 mmol) was added to a 25 ml solution of 5-(4-dodecyloxyphenyl)dipyrromethane (500 mg, 1.23 mmol) in toluene. The reaction mixture was kept in the dark and stirred at room temperature. Following 5 minutes of stirring, TLC (silica gel, 20% ethyl acetate/hexane) showed the consumption of the starting dipyrromethane at which point triethylamine (1.2 ml, 8.61 mmol) was added followed immediately by boron trifluoride etherate (1.1 ml of neat BF_3-etherate, 8.61 mmol). The reaction mixture was stirred at room temperature for 1 hour, decanted from the dark green sludge which was washed with toluene (10 ml) and the combined toluene extracts washed with water, dried (Na_2SO_4) and evaporated to give a dark-green viscous oil. This was purified by column chromatography (silica gel, DCM, R_f 0.54) to give compound **1** as a red-orange crystalline solid. Yield 0.19 g (34 %): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (s, 2H, pyrrole-H); 7.55 (d, 2H, *J* = 8.6 Hz, phenyl-H); 7.05 (d, 2H, *J* = 8.6 Hz, phenyl-H); 7.01 (d, 2H, *J* = 4.1, pyrrole-H); 6.57(m, 2H, pyrrole-H); 4.07 (t, 2H, *J* = 6.5 Hz, O-**CH_2**); 1.86 (m, 2H, 0-CH_2-**CH_2**); 1.57-1.29 (m, 18H, -**CH_2**-); 0.91 (t, 3H, 6.6 Hz, -**CH_3**); ^{13}C NMR (400 MHz, $CDCl_3$) δ 161.78,

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147.57, 143.31, 134.84, 132.46, 131.36, 126.09, 118.24, 114.53, 68.37, 31.93, 29.69, 29.60, 29.38, 29.16, 26.04, 22.71, 14.15. MS (TOF-ES) 475.3 (MNa^+)

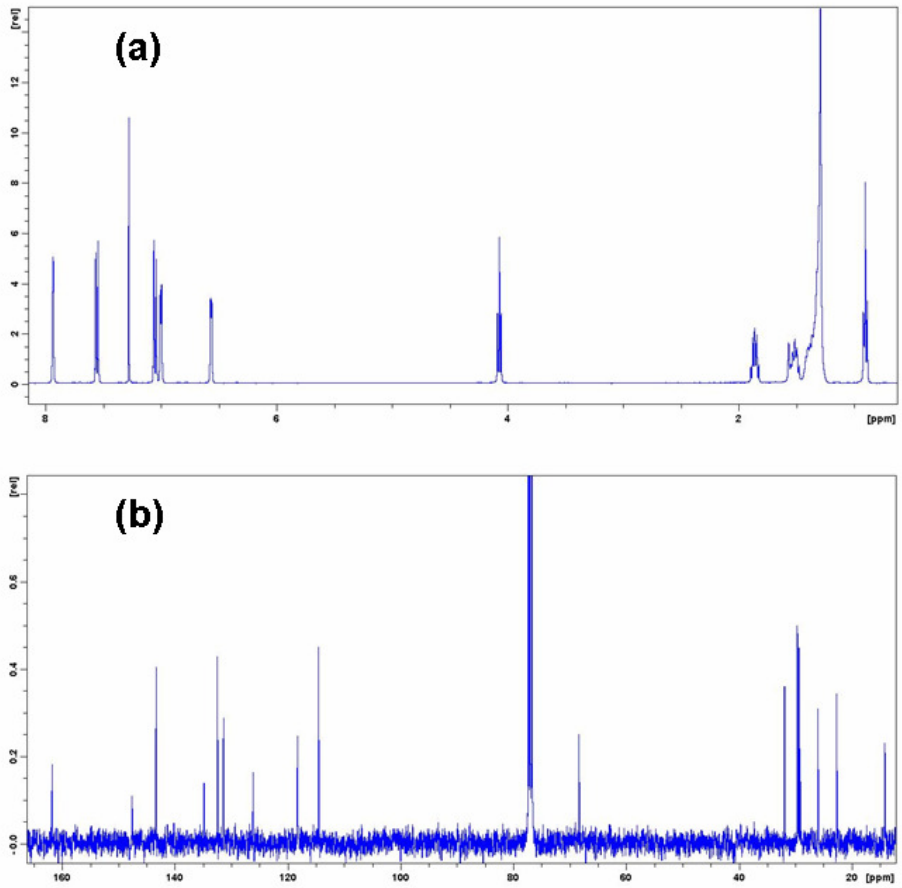

Figure S1: ^{1}H NMR (a) and ^{13}C NMR (b) spectra of compound **1.**

Fluorescence imaging

The human ovarian carcinoma cell line SK-OV-3 was obtained from the European Collection of Cell Cultures (ECACC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum, penicillin and streptomycin antibiotics and passaged when 70-90% confluent in 75 cm^3 flasks grown at 37ºC in 5% CO_2. For imaging SK-OV-3 cells were seeded at 10^4 cells/well in 0.2 ml of culture medium in untreated 8-well coverglass chambers (Lab-TekTM, Nunc) and allowed to grow to confluence for 24 h. The culture media was replaced with the culture medium containing **1** and incubated for 30 min. Following incubation, the chambers were washed twice with PBS and images taken at 25°C.

Imaging was performed using a confocal laser scanning microscope (Leica TCS SP2), coupled to a CW argon-ion laser (488 nm). The fluorescence emission of **1** from cells was spectrally dispersed using a prism and detected using a PM tube. Water immersion 63× objective (NA = 1.2) was used in all measurements.

FLIM

FLIM images of 256×256 pixels were obtained using a Leica TCS SP2 inverted scanning confocal microscope coupled with a Becker & Hickl time-correlated single photon counting (TCSPC) card SPC830 in a 3GHz, Pentium IV, 1GB RAM computer running Windows XP. A pulsed diode laser at 467 nm (PLP-10 470, Hamamatsu) with a pulse duration of 90 ps and a repetition rate of 20 MHz was used as the excitation source. Imaging was carried out with a 63× water immersion objective (NA = 1.2) and a line scanning speed of 400 Hz. The emission was collected through a 525±25 nm band pass filter onto a cooled PMC 100-01 detector (Becker & Hickl, based on a Hamamatsu H5772P-01 photomultiplier). The acquisition time was 200 s for each image.

A single exponential decay was fitted to the data to find the average fluorescence lifetime of **1**. SPCImage software 2.8 (Becker & Hickl) based on a Levenberg-Marquardt fitting algorithm was used to fit a mono-exponential decay to the fluorescence decay curve in each pixel of the image, using an instrumental response function generated by the fitting software from the rising edge of the decay. A false colour scale was assigned to each fluorescence lifetime value (red for a short lifetime and blue for a long lifetime), yielding FLIM maps.

The fluorescence decays from solution samples were recorded using the same set up as for the imaging experiments using the SPC software in the oscilloscope mode. The methanol/glycerol solutions of various viscosities were prepared in triplicate and the resulting values for fluorescence lifetime and rotational correlation time averaged.
We define the time resolved fluorescence anisotropy *r(t)* as

$$r(t) = \frac{I_{||}(t) - G \times I_{\perp}(t)}{I_{||}(t) + 2G \times I_{\perp}(t)} \qquad \text{(Equation S1)}$$

Where $I_{||}(t)$ and $I_{\perp}(t)$ are the fluorescence intensity decays parallel and perpendicular to the polarization of the exciting light. *G* accounts for different detection and transmission efficiencies of imaging system at parallel and perpendicular polarization[2] and was established to be equal to 1.0 in the present measurements using either a standard sample of fluorescein or the tail matching of the decays with parallel and perpendicular polarisation. For a spherical rotor with restricted rotational mobility, *r(t)* decays monoexponentially according to:

$$r(t) = (r_0 - r_\infty)\exp\left(-\frac{t}{\theta}\right) + r_\infty \qquad \text{(Equation S2)}$$

Where r_0 is the initial anisotropy and r_∞ is the limiting anisotropy resulting from restricted rotational mobility.
We have calculated the *r(t)* for a series of glycerol/methanol mixtures of various viscosity from the experimental data for $I_{||}(t)$ and $I_{\perp}(t)$ decay according to Eq. S1 and obtained the series of θ from a monoexponential fit to these data (r_∞=0). No deconvolution analysis was necessary for these decays as the instrument response function is ca 200 ps, significantly shorter than the fastest rotational correlation time decay, *ca* 1 ns. For a spherical rotor in an isotropic medium θ is directly proportional to the viscosity of the solvent and volume V of the rotating molecule, equation 2, thus the plot of θ vs η allows the calculation of the volume of the molecular rotor. k is the Boltzmann constant, and T the absolute temperature.

Viscosity measurements
The viscosity of methanol/glycerol mixtures was measured using an advanced rheometric expansion system rheometer (ARES)[3] equipped with a force-rebalance transducer (torque range, 4×10^{-8}–1×10^{-3} Nm). A double-walled Couette-type rheometric tool was used for the measurements (cup outer radius, 17.00 mm; cup inner radius, 13.98 mm; bob outer radius 16.00 mm; bob inner radius, 14.75 mm, resulting in 1 mm external gap and 0.77 mm inner gap. The temperature was kept at (22.0±0.1)°C with a bath circulator (Julabo F33). Each measurement used ca. 7 ml of sample. The viscosity was measured as a function of shear rate in the range from 1 to 100 s^{-1} and was found to be constant.

(2) Photophysical parameters of 1 in the methanol/glycerol mixtures of varied viscosity
The non-radiative (k_{nonrad}) and radiative decay constants (k_0) were calculated from experimentally measured fluorescence quantum yield ϕ and fluorescence lifetime τ according to the following equations:

$$\phi = \frac{k_0}{k_0 + k_{nonrad}} \qquad \tau = \frac{1}{k_0 + k_{nonrad}} \qquad \text{(Equation S3)}$$

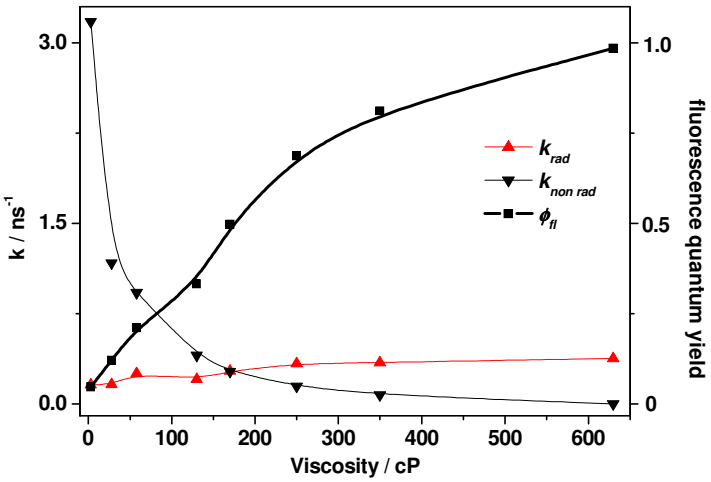

Figure S2: Fluorescence quantum yields, non-radiative and radiative decay constants for **1** obtained in methanol/glycerol mixtures of varied viscosity. The curves are guides for the eye.

(3) Intracellular localization of 1

The confocal fluorescence image of the living cells (SK-OV-3 epithelial adenocarcinoma cell line) incubated with **1** is shown in Figure 3a (main manuscript text) and clearly shows the intracellular uptake of the molecular rotor with punctuate dye distribution. The punctuate fluorescence pattern is typical of mitochondria and vesicular organelles, such as lysosomes, peroxisomes, endosomes. Our costaining experiments with Lysotracker Red® (Molecular Probes, staining lysosomes) do not show significant overlap with the fluorescence of **1**. Endocytotic uptake of **1** into SK-OV-3 cells can lead to localisation in the endosomes and this was tested in the variable temperature uptake experiments.

To determine the mechanism of the cellular uptake of **1** we have monitored the fluorescence of the confluent layer of cells incubated with **1** for 30 min at either 37°C or at 0°C (on ice). In the latter case the endocytotic uptake pathway is arrested, while passive diffusion remains active.

The fluorescence images recorded after 37°C and 0°C incubation experiments are compared in Fig S3 and clearly show that the intracellular uptake of **1** is significantly decreased at 0°C incubation. In addition the continuous monitoring of the intracellular uptake of **1** at 25°C on the microscope stage from 1 to 40 min incubation shows that the distribution of **1** inside cells does not change as a function of the incubation time, but the fluorescence intensity increases. This result indicates that there are no additional cellular organelles involved in the uptake of **1**, other than those apparent at images following 30 min incubation. We therefore conclude that **1** is taken up by SK-OV-3 cells mainly by endocytosis and the primary organelles targeted are endocytotic vesicles.

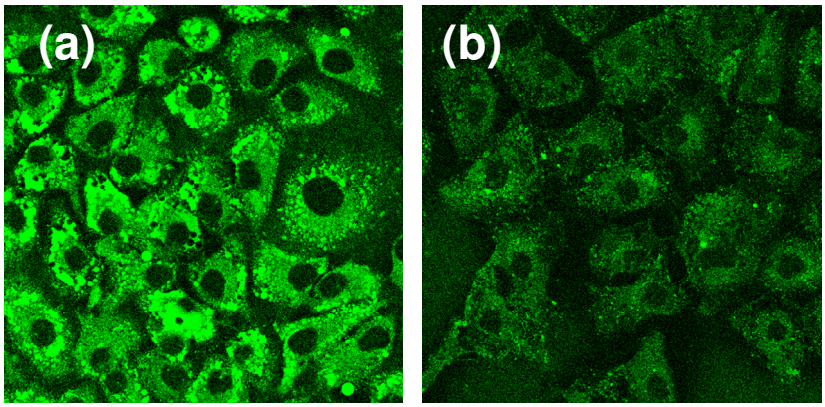

Figure S3: Confocal fluorescence images obtained following 488 nm excitation and (525±50) nm detection from SK-OV-3 cells incubated with 1 μM solution of **1** for 30 min (a) at 37°C and (b) at 0°C on ice.

(4) FLIM study of 1 in SK-OV-3 cells

The FLIM image obtained following single photon excitation of **1** in SK-OV-3 cells is shown in Fig S4. We find that these data are well fitted with monoexponential decay function, Fig. S4.

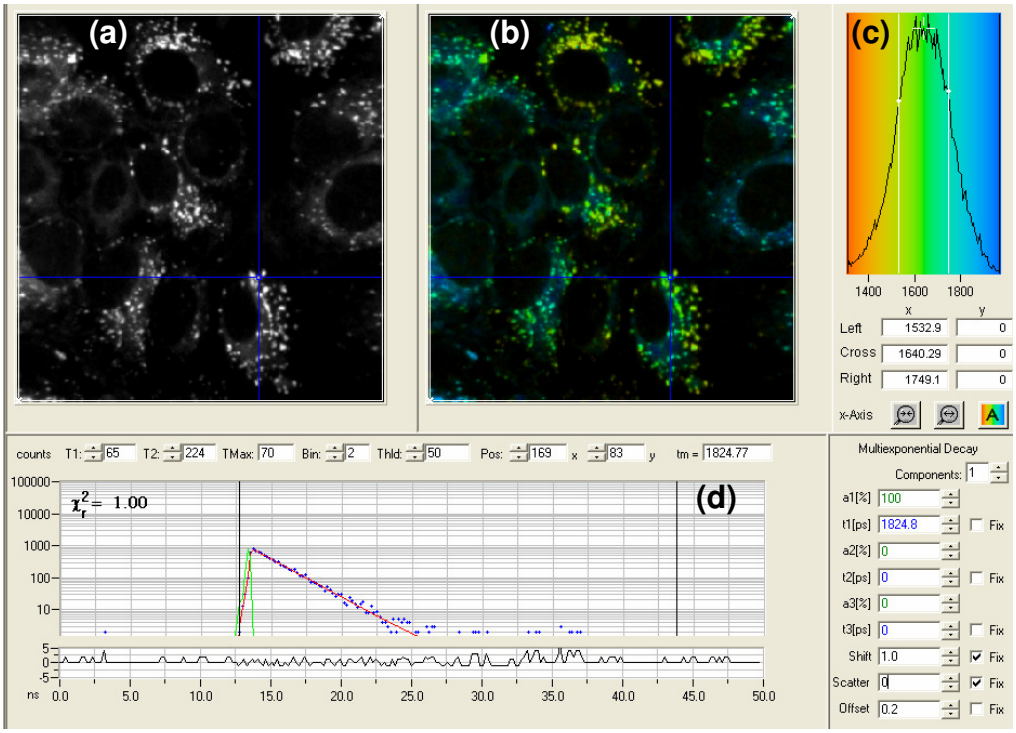

Figure S4: (a) Fluorescence image obtained following 467 nm excitation and (525±25) nm detection from SK-OV-3 cells incubated with 1 μM solution of **1** (b) FLIM image obtained following 467 nm pulsed excitation of the same layer of cells (c) histogram of lifetimes obtained from the fit and (f) typical decay trace.

The histogram of the fluorescence lifetime distribution inside SK-OV-3 cells gives the value of (1.6±0.2) ns, Fig S4 (c). On the lifetime calibration graph, Fig 2a, this lifetime range corresponds to the viscosity value of (140±40) cP.

However we note that the width of the lifetime distribution does not necessarily correspond to a broad distribution of viscosities. Even a uniform viscosity in a homogenous sample will produce a statistical distribution of lifetimes. The width of this distribution depends on the number of photons in the decays - the larger number of photons leading to narrower distributions. For homogenous solution samples with ca 1000 photons in the decay maxima (close to cell sample conditions) we expect the histogram width of ca 2.5%, *e.g.* (4.0±0.1) ns (fluorescein). The histogram of the fluorescence lifetime distribution inside cells gives the value of (1.6±0.2) ns, which gives a wider distribution than in a homogenous solution sample and this might be expected in a heterogenous sample such as cells with the wider range of the fluorophore environments.

(5) Anisotropy decay of 1 in SK-OV-3 cells

The anisotropy decays were measured in several locations within the cell layer (>5 measurements), each trace fitted to a monoexponential decay and the resulting values averaged to yield (1.1±0.3) ns as an average over the whole cell. The typical anisotropy decay of **1** is given in Fig S5

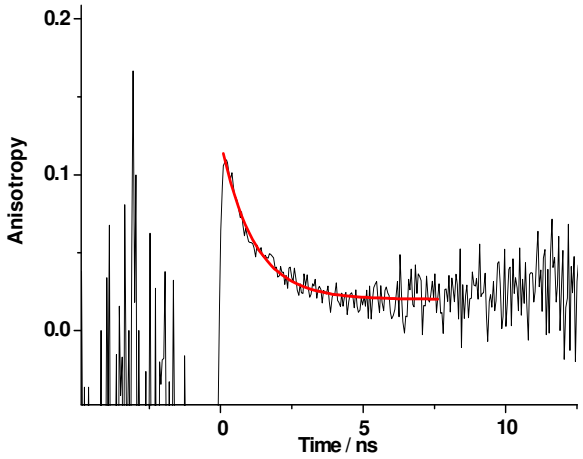

Figure S5: The typical anisotropy decay of **1** obtained from the layer of SK-OV-3 cells. A monoexponential fit yields θ=(1.0±0.1) ns

(6) Consideration of the secondary effects on the photophysics of 1 in cells

For the applications of **1** as a bioviscosity sensor it is essential that its fluorescence lifetime is not affected by other environmental factors such as pH, dielectric constant, ionic strength, polarity of the solvent and the presence of the excited state quenchers. It is well known that the spectral characteristics of unmodified BODIPY chromophores, including lifetimes, are largely independent on the solution pH and polarity of the solvent.[4,5] The quenching of BODIPYs with protein components, *e.g.* tryptophan (Stern Volmer quenching constant of 15 M^{-1}), tyrosine (K=14 M^{-1}) and phenylalanine (K=1.4 M^{-1}) has been investigated[4] and this study concluded that such quenching requires high concentration of the quencher and leads to non-monoexponential excited state decays of BODIPY, which is not observed in our study.

Additionally, the presence of the secondary excited state processes can be tested by correlating the change of rotational correlation time θ with fluorescence lifetime τ. θ is independent of solution ionic strength, the presence of quenchers or pH. Hence in the absence of these effects on the fluorescence lifetime the following equation should hold:

$\tau = a\,\theta^{\alpha}$

where a is a constant.

A plot of log(τ) *vs* log(θ) is given in Fig S6 and shows a linear correlation confirming that τ is not affected by secondary processes in our experiments. In the cell experiments both τ and θ calibration graphs yield the cellular viscosity value of the same magnitude, see Fig S6 (red).

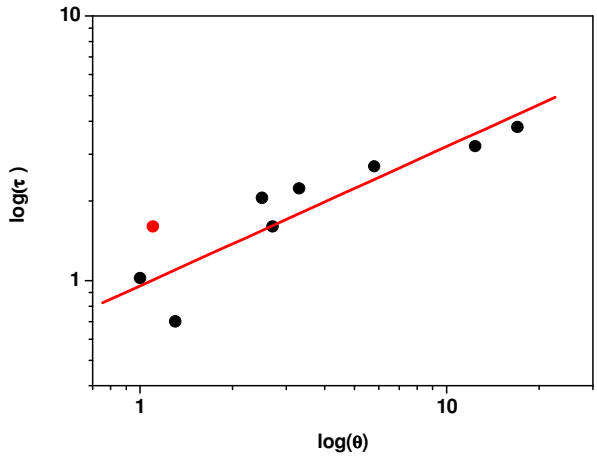

Figure S6: Correlation graph of τ and θ for **1,** according to $\tau = a\theta^{\alpha}$, obtained in methanol/glycerol mixtures of varied viscosity. The value obtained in SK-OV-3 cells is shown in red.

We have also demonstrated that **1** does not form aggregates at the conditions used in the present study. It is known that at high concentrations BODIPY forms aggregates which are characterised by the emission maxima at >630 nm (for the unmodified chromophore)[6] and are probably characterised by much shorter excited state lifetime. We do not observe any emission from cells incubated with **1** at >600 nm and do not observe a shorter lifetime component in fluorescence decay of **1** in cells, which confirms the absence of the aggregates.

7 References

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