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Characterization of changes in the viscosity of lipid membranes with the molecular rotor FCVJ

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Abstract

Membrane viscosity is a key parameter in cell physiology, cell function, and cell signaling. The most common methods to measure changes in membrane viscosity are fluorescence recovery after photobleaching (FRAP) and fluorescence anisotropy. Recent interest in a group of viscosity sensitive fluorophores, termed molecular rotors, led to the development of the highly membrane-compatible (2-carboxy-2-cyanovinyl)-julolidine farnesyl ester (FCVJ). The purpose of this study is to examine the fluorescent behavior of FCVJ in model membranes exposed to various agents of known influence on membrane viscosity, such as alcohols, dimethyl sulfoxide (DMSO), cyclohexane, cholesterol, and nimesulide. The influence of key agents (propanol and cholesterol) was also examined using FRAP, and backcalculated viscosity change from FCVJ and FRAP was correlated. A decrease of FCVJ emission was found with alcohol treatment (with a strong dependency on the chain length and concentration), DMSO, and cyclohexane, whereas cholesterol and nimesulide led to increased FCVJ emission. With the exception of nimesulide, FCVJ intensity changes were consistent with expected changes in membrane viscosity. A comparison of viscosity changes computed from FRAP and FCVJ led to a very good correlation between the two experimental methods. Since molecular rotors, including FCVJ, allow for extremely easy experimental methods, fast response time, and high spatial resolution, this study indicates that FCVJ may be used to quantitatively determine viscosity changes in phospholipid bilayers.

Keywords: Viscosity; Fluidity; FRAP; Molecular rotor; Alcohol; Cholesterol; Fluorescence

1. Introduction

Membrane viscosity has been used as a parameter to characterize the ease of movement of a particle within the two dimensional realm of the cell membrane [1]. The proper function of membrane proteins is directly dependent on local viscosity. Changes in membrane viscosity, or its reciprocal, fluidity, have been linked with disease states and have been proven as useful indicators of cell viability [2–5]. Changes in carrier mediated transport and membrane-bound receptors have all been correlated with changes in membrane viscosity [6,7]. Furthermore, increases in membrane viscosity have been reported with the onset of atherosclerosis [4], malignancy [8], diabetes [9,10], and hypercholesterolemia [11]. Conversely, an increase in membrane fluidity has been linked with amyloid precursor protein production in Alzheimer's patients [12]. Alcohols and anesthetics are excellent examples to demonstrate the influence of some drugs on membrane viscosity. The viscosity-reducing effect of alcohols is particularly well explored [13–15], while some anesthetics, applied at high concentrations, decrease membrane viscosity by unspecifically binding to the membrane [13,16].

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There are a number of methods that have been developed to monitor changes in membrane viscosity. Mechanical methods, such as micropipette aspiration were first to be introduced. This technique measures the deflection of an aspirated membrane under known pressure gradients over time. The deflection rate can then be used to calculate the apparent viscosity through nonlinear regression [17]. One drawback of this method is that membrane viscosity must be extrapolated as a function of external physical parameters. In addition, membrane measurements can take several seconds to minutes. Magnetic rheometry is a technique that allows to examine local viscosity changes. In this technique magnetic beads are attached to the membrane and exposed to a uniform magnetic field. Viscosity restricts the movement of the particles in the magnetic field, so inferences about local membrane viscosity can be made [18]. Both of these methods have significant temporal and spatial limitations.

Fluorescent probes are a popular alternative to estimate membrane viscosity. Currently there are two well-established methods for measuring membrane viscosity: (a) fluorescence recovery after photobleaching (FRAP) which measures diffusivity of specific membrane-bound fluorescent dyes [19], and (b) fluorescence anisotropy, which measures depolarization of fluorophores excited by polarized light, where depolarization depends on membrane fluidity [20].

A more recent approach to assess membrane viscosity is the use of fluorescent molecular rotors, a group of fluorophores known to be sensitive to the free volume and thus the viscosity of their environment [21]. Molecular rotors belong to a group of fluorescent molecules, defined as twisted intermolecular charge transfer (TICT) complexes [22] that have two deexcitation pathways. A molecular rotor either assumes the twisted state with subsequent nonradiative deexcitation, or it emits a photon. Since TICT formation is hindered in environments of higher viscosity, the quantum yield increases. More specifically, the quantum yield ϕ_F depends on the local microviscosity η according to Eq. (1) [23]:

$$\log\phi_F = C + x\cdot\log\eta \quad (1)$$

In this equation, C and x are proportionality constants, where typically $x=0.6$, and all values are taken to be unitless numbers. Fluorescence emission intensity is proportional to ϕ_F, therefore simple intensity measurements allow an estimate of microviscosity changes. The farnesol ester of the molecular rotor (2-carboxy-2-cyanovinyl)-julolidine, abbreviated FCVJ (Fig. 1), has been introduced as a particularly membrane-compatible fluorophore [24].

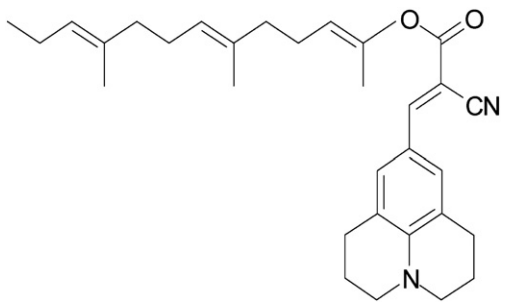

Fig. 1. Chemical structure of the molecular rotor (2-carboxy-2-cyanovinyl)-julolidine farnesyl ester (FCVJ).

The cell membrane is a highly inhomogeneous, complex physical system. Viscosity, or its reciprocal, fluidity, has been used to describe diffusivity, packing, and permeability of membranes [25]. Furthermore, protein activation has been linked to membrane viscosity [26]. Artfully designed fluorescence probes, *n*-(9-anthroyloxy) fatty acids, allow to examine the heterogeneity of the bilayer as a function of depth [27], particularly to determine fluidity gradients [28]. The reported apparent viscosity is clearly dependent on the exact location of the probe. For these reasons, the notion of membrane viscosity is not directly comparable to the bulk viscosity of fluids. Nonetheless, the notion of membrane fluidity (or viscosity) is useful in characterizing the dynamic properties of the membrane [29,30].

The goal of this study was to examine the emission behavior of FCVJ entrapped in liposome model membranes when the membranes were exposed to viscosity-altering agents. Agents known to reduce membrane viscosity, such as alcohols [31], and agents known to increase membrane viscosity, such as cholesterol [1] were examined. For another agent, nimesulide, conflicting reports exist. In some studies, a decrease in membrane viscosity was reported [32,33], whereas another group did not observe any viscosity changes [34].

It was also the goal of this study to examine the ability of FCVJ to accurately report membrane viscosity changes through fluorescence spectroscopy, and to compare FCVJ emission changes to recovery dynamics using FRAP in representative cases.

2. Materials and methods

2.1. Liposome formation

All agents, alcohols and solvents listed in this and the following sections were purchased from Sigma-Aldrich unless otherwise stated. FCVJ was synthesized by our group [24]. A volume of 20 μL FCVJ stock solution (2.5 mM FCVJ in spectroscopy-grade dimethylsulfoxide (DMSO) was added to 200 μL of a 10 mg/mL solution of DLPC:chloroform (Avanti Polar Lipids) in a glass vial. Established electroformation procedures were used to create unilamellar liposomes [35,36]. Briefly, a glass syringe was used to deposit the chloroform solution of DLPC and FCVJ onto platinum electrodes in an electroformation chamber. The chamber was placed under vacuum for 30 min to remove organic solvent. Glass slides were attached with vacuum grease (Dow Chemicals) to enclose the chamber. The chamber was then flooded with 1.5 mL of 250 mM aqueous sucrose solution to act as the formation solution. The formation process was observed under epifluorescent microscopy. A 10 Hz, 1 V_{pp} sinusoidal AC electric signal was applied to the two electrodes for 10 min. The frequency was then lowered to 1 Hz and allowed to run for an additional 7 min. The final liposome suspension was extracted with a syringe and stored in a microcentrifuge tube on ice.

2.2. Experiments with short-chain alcohols and organic solvents

A volume of 50 μL of the liposome suspension was pipetted into 950 μL of the stock sucrose solution in a microcuvette. The solution was then briefly vortexed. The cuvette was placed in temperature controlled turret (Quantum Northwest) set at 30 °C. The sample was then excited at 460 nm in the spectrofluorometer (Fluoromax-3, Jobin-Yvon). Emission spectra were gathered from 470 nm to 600 nm. Slit settings of 5 nm were chosen to achieve peak emission intensities of less than 2×10^6 photon counts per second to avoid detector saturation. This procedure was repeated ten times with fresh diluted liposome suspension and used as the control group. The experimental group

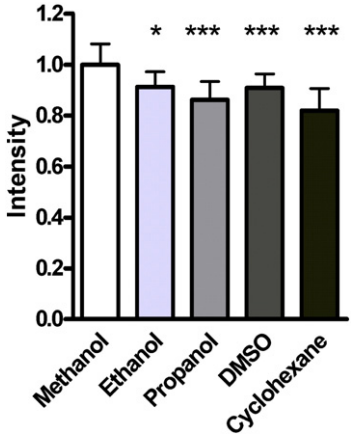

Fig. 2. Intensity changes caused by the introduction of various solvents (alcohols at 2% v/v, DMSO and cyclohexane at 5% v/v) to the liposome suspension. All samples were normalized with respect to their relative controls. While the addition of methanol did not cause a significant decrease in intensity, peak intensity was significantly reduced over the control groups.

consisted of altering the carrier fluid with a variety of alcohols and organic solvents. The experimental group was formed by adding 50 μL of the liposome suspension to 950 μL of 2%(v/v) pure methanol in aqueous sucrose solution. This procedure was repeated for ethanol, propanol, and cyclohexane at identical concentrations. The DMSO experimental set consisted of adding 50 μL of liposome suspension to 950 μL of 30 mol% DMSO in aqueous sucrose solution. This procedure was repeated 10 times for each experimental agent.

2.3. Experiments with cholesterol and nimesulide

Due to the hydrophobic nature of the cholesterol and nimesulide, these two agents were added to the phospholipid/dye mixture prior to formation. Liposomes containing the agent were prepared by adding either 10 μL of a 10 mM nimesulide solution in methanol or 20 μL of a 20 mol% solution of cholesterol in chloroform to 180 μL of a solution of DLPC in chloroform. 10 μL of 2.5 mM FCVJ stock solution was added into this solution. The electroformation process was performed as described in Section 2.1. Ten samples containing 50 μL of either nimesulide liposomes or cholesterol liposomes in 950 μL sucrose (250 mM) were used as the experimental groups, and fluorescence spectra were acquired as described above. A separate batch was prepared for use as the control group without the addition of nimesulide or cholesterol. The excitation and emission settings were identical to those described in Section 2.1.

2.4. FRAP experiments

Supported lipid bilayers were formed via vesicle fusion inside PDMS wells adhered to clean glass slides [37]. The lipid bilayers were then incubated in solutions containing 0%, 0.5%, 1% and 2% propanol in water. Additional bilayers were prepared with 15 mol% of cholesterol. FRAP was then performed as previously described [37] at four different spots on the bilayer for each concentration respectively. Diffusion coefficients D were calculated in $\mu m^2\ s^{-1}$ through Eq. (2),

$$D = 0.224 \cdot \frac{r^2}{t_{1/2}} \tag{2}$$

where r is the radius of the bleached spot in μm and $t_{1/2}$ is the halftime of recovery of the fluorescence recovery in s [19,38]. The value of $t_{1/2}$ was obtained through nonlinear regression of the recovery intensity as a function of time with an exponential association. Viscosity is inversely proportional to D [19,38].

The resulting diffusion coefficients were averaged over the four spots for each concentration. By using the propanol-free medium (0%) as a control, a change in viscosity was calculated for each of the propanol concentrations.

2.5. Data analysis

Fluorescence peak emission data was gathered for each sample and the mean was determined for each group. Relative viscosity was calculated using Eq. (3) [39].

$$\left(\frac{\eta_2}{\eta_1}\right) = \left(\frac{I_2}{I_1}\right)^{\frac{1}{x}} \tag{3}$$

In Eq. (3), I_1 is the peak intensity of the control group or of the sample before treatment, I_2 is the peak intensity of the treatment group, η_2/η_1 is the relative change of viscosity, and $x=0.6$. If background light is negligible and temperature and experimental conditions are identical for all samples, this equation eliminates the constant C that was introduced in Eq. (1) [39].

To allow better comparison between groups, all peak intensities were normalized by the mean intensity of their respective control group. The normalized mean peak intensity of the treatment group was then compared to the normalized mean peak intensity of the control group through either Student's t-test or one-way ANOVA depending whether a comparison of one treatment group to the control was needed, or a comparison of multiple groups, respectively. The bar graphs show the normalized mean intensity, and error bars indicate standard deviation. Statistical significance is indicated by stars where * indicates $P<0.05$, ** indicates $P<0.01$, and *** indicates $P<0.001$. Graphpad PRISM, version 4.01, was used for data analysis.

In the FRAP experiments, relative viscosity was computed using Eq. (4),

$$\left(\frac{\eta_2}{\eta_1}\right) = \left(\frac{1/D_2}{1/D_1}\right) \tag{4}$$

where D_1 and D_2 were the diffusion constants of the control and treatment groups, respectively. This change in viscosity was plotted against the viscosity change observed in the FCVJ model system and linear regression was performed to obtain the correlation coefficient between FCVJ- and FRAP-derived relative viscosities.

3. Results and discussion

3.1. Short chain alcohols

It has been shown that short-chain alcohols localize predominantly at the hydrophilic headgroup region of the phospholipid bilayer. Their location disturbs the natural microstructure of the lipid membrane leading to a decrease in membrane viscosity. Moreover, the size of the hydrocarbon chain of the alcohols was found to be approximately proportional to the observed viscosity decrease [31]. Quantitatively, this trend follows Traube's rule of interfacial tension reduction [40] which predicts that, for every additional methyl group of its hydrocarbon sidechain, an alcohol becomes three times more effective in decreasing the

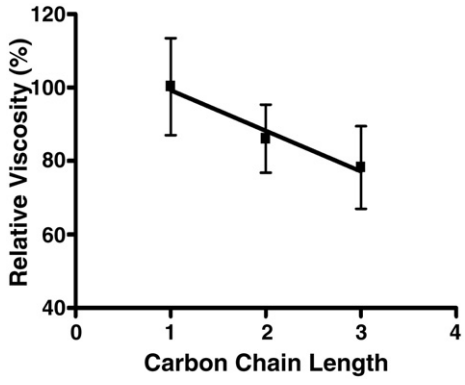

Fig. 3. Viscosity change as a function of carbon chain length of the alcohols methanol, ethanol, and propanol. All alcohols were present at a concentration of 2% (v/v).

interfacial tension of the bilayer. The studies shown in Figs. 2 and 3 demonstrate clearly that FCVJ can accurately report decreases in membrane viscosity that are proportional to the size of the hydrocarbon sidechains of the alcohols tested. Fig. 2 shows the mean intensities of FCVJ peak emission in liposomes exposed to various alcohols and the solvents cyclohexane and DMSO. The effect of methanol on the membrane system is minimal (<1%) and statistically not significant. Exposure to 2% v/v ethanol causes a reduction in mean intensity of 8.7%. Continuing with the trend, propanol causes a decrease of nearly 13.8% in intensity. Using Eq. (3), viscosity reductions to 87% (ethanol) and 81% (propanol) of the control value were calculated. This increasing effect of carbon chain length of the alcohol on intensity is in agreement with Traube's rule. Fig. 3 shows decrease in viscosity (calculated using Eq. (3)) as a function of carbon chain length with a proposed linear correlation ($R^2=0.98$). It is unlikely that this linear trend would hold for very long carbon chains and it would be of interest in further studies to examine the exact relationship between alcohol chain length and membrane viscosity.

3.2. Effect of propanol concentration on membrane viscosity

Not only the chain length, but also the alcohol concentration has an influence on membrane viscosity. It has been demonstrated that alcohol concentration increases the density of the alcohol at the phosholipid surface [31] which leads to decreased membrane viscosity. To demonstrate that FCVJ accurately reports those changes, propanol was chosen for its strong effect on the membrane. The relationship between FCVJ intensity and propanol concentration is shown in Fig. 4. As expected, a decrease in intensity is observed with an increase in propanol concentration. Linear regression of computed viscosity as a function of propanol concentration exhibits a good correlation ($R^2=0.91$), but nonlinear models may better describe the relationship.

3.3. Organic solvents

DMSO has been reported to be responsible for the formation of pores for water flux within the membrane leading to a decrease in viscosity [41]. Accordingly, DMSO produced a reduction in mean intensity of FCVJ emission in the FCVJ-stained liposomes, thus reporting the decrease in viscosity. With an approximate DMSO concentration of 5% v/v, an intensity decrease of 9.2% (corresponding to a reduction of viscosity to 86% of its original value) was observed (Fig. 2) and was found to be statistically significant ($P=0.0005$).

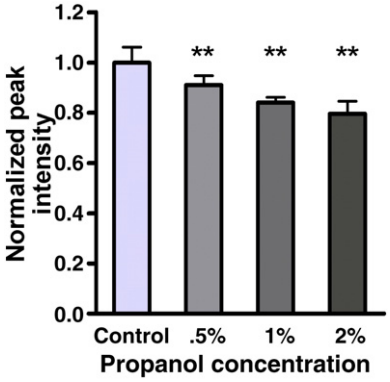

Fig. 4. Dose response of FCVJ emission intensity to increasing concentrations of propanol. With concentrations as low as 0.5%, statistically significant changes of intensity can be seen, and higher propanol concentrations cause lower emission intensities.

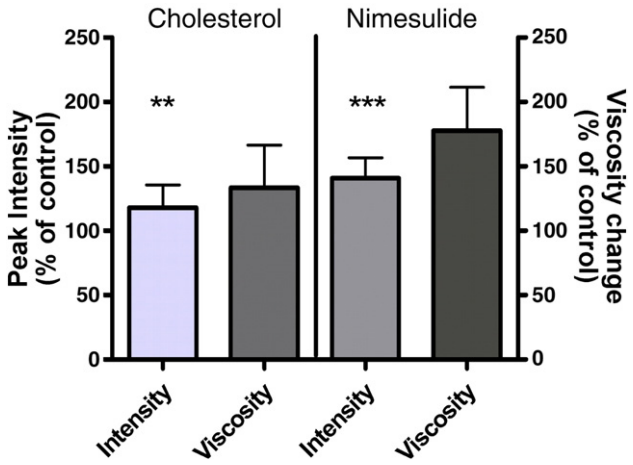

Fig. 5. Intensity change and computed viscosity change as a result of the addition of the hydrophobic agents cholesterol (left) and nimesulide (right), respectively. The addition of cholesterol to the liposome formation media induced a statistically significant increase in emission intensity of 18% corresponding to a viscosity increase of 32% ($P<0.01$), whereas the nimesulide induced a 41% intensity increase corresponding to a 63% increase in viscosity ($P<0.0001$) compared to the control group.

Cyclohexane is an organic solvent with very limited solubility in water. It has been shown that at low concentration of this solvent, the polar water molecules can orient around it to form "water cages" [1] which promote the partitioning of cyclohexane into the liposomes. The addition of approximately 5% v/v of cyclohexane to the liposome suspension was found to cause a reduction in intensity of 18.2% (corresponding to a reduction of viscosity to 76% of its original value) as shown in Fig. 2. The effects of cyclohexane on the membrane are profound and statistically significant ($P=0.0001$).

3.4. Cholesterol and nimesulide

Cholesterol was chosen for its role in a number of physiological processes [1]. Cholesterol is a key component in regulating membrane viscosity. Cholesterol acts as a spacer in the membrane, and an increase in membrane viscosity is expected upon integration into the membrane [1].

Nimesulide is a COX-2 inhibitor commonly prescribed as arthritis medication. The drug interrupts the prostaglandin synthesis pathway and in turn reduces inflammation. The action of the drug on the cell membrane is not well understood [32]. Recently, gastric side effects have been reported with the use of this drug. It has been reported that nimesulide tends to migrate towards the interior of the phospholipid bilayer, possibly causing a decrease of membrane viscosity [32,33]. Fig. 5 highlights the results from both of these experiment sets. The added cholesterol was shown to induce an 18% increase in intensity (32% increase in viscosity) whereas the nimesulide induced a 41% increase

in intensity (63% increase in viscosity). Both the cholesterol and nimesulide effects were found to be statistically significant with $P=0.001$ and $P=0.0001$ respectively. Whereas the intensity increase associated with cholesterol was expected, conflicting results on the effects of nimesulide exist. Previous reports revealed that nimesulide can reduce membrane viscosity (i.e., increase fluidity) [33], other reports indicated no significant change [34], while a recent report showed that both observations can be correct, depending on the concentration of nimesulide. For instance, exposing egg-PC bilayers to a concentration of 50 μM nimesulide reduced the membrane viscosity, while exposure to 100 μM nimesulide strongly increased viscosity compared to membranes in an aqueous solution without nimesulide [37]. In addition, free radicals may be involved in the anisotropy change [42] to which FCVJ might be less susceptible. In experiments involving this specific agent, additional mechanical validation methods would be helpful.

3.5. Comparison to FRAP

FRAP is an established method that has been shown to effectively measure membrane viscosity through membrane diffusion measurements [43]. With the known relationships between FCVJ emission intensity and viscosity as well as FRAP diffusivity and viscosity (Eqs. (3) and (4), respectively), a correlation between viscosity changes observed through FCVJ and FRAP was established. Fig. 6 shows the measured change in viscosity for the FCVJ system compared to using traditional FRAP in supported lipid bilayers. The good linear correlation ($R^2=0.96$) suggests an excellent agreement between FRAP and FCVJ. However, the slope of the linear fit was found to be 1.7, indicating that – relative to FRAP – the molecular rotor either consistently overestimates the viscosity change or exhibits a higher sensitivity. FRAP experiments showed that cholesterol decreases diffusivity by 26% and therefore increases membrane viscosity by 36%. This is in good agreement with the 32% viscosity increase found using FCVJ.

There are two factors that may explain the difference between FRAP and FCVJ in estimating viscosity. First, both methods derive viscosity indirectly. FRAP is a method to measure fluorophore diffusivity, whereas a molecular rotor reports the molecular free volume [44] of its environment. It can be assumed that the agents acting on the membrane change both parameters in different ways. Second, FCVJ and FRAP use highly different time scales. A molecular rotor responds to changes in its environment within the excited-state lifetime of several hundred picoseconds. FRAP requires 6 to 9 min for the photobleaching process [37] with up to 30 min of recovery time. During the photobleaching process, dye will already start migrating into the irradiated spot. Over the bleaching time, a zone of reduced dye concentration will form around the bleached spot that slows the recovery process. Higher diffusivity can be thought to increase the radius of the dye-depleted zone, thereby slowing the recovery after photobleaching and therefore reducing the apparent differences between the untreated membrane and a membrane treated with a viscosity-reducing agent. Moreover, FRAP reports diffusivity of membrane components over a relatively large area (in this case, a spot of 30 μm [37]) whereas FCVJ reports free volume in the area immediately surrounding the rotors. Under microscopic resolution, it can be expected that a molecular rotor is capable of reporting the impact of a compound (such as a protein) on local membrane free volume whereas FRAP is restricted to reporting the diffusivity average over a wider area.

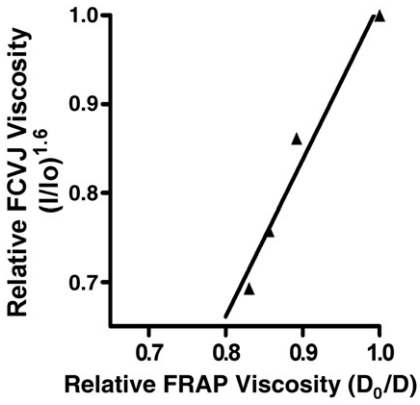

Fig. 6. FCVJ derived viscosity versus FRAP-derived viscosity of phospholipid bilayers exposed to propanol concentrations of 0%, 0.5%, 1%, and 2% (v/v). Both methods show a good linear correlation with $R^2=0.96$.

4. Conclusion

We have demonstrated that the molecular rotor FCVJ can be successfully integrated into the lipid bilayer. Furthermore, the rotor exhibited sensitivity to viscosity observed through changes in its fluorescence emission intensity. Molecular rotors react almost instantly to viscosity changes in their environment, and they report changes in viscosity at a high spatial resolution [45]. Molecular rotors have been used to report conformational changes in proteins [46,47], polymerization processes [44], and even cell membrane viscosity changes [39]. Particularly in cells, however, commercially available molecular rotors, such as 2-(dicyanovinyl)-julolidine (DCVJ) have a tendency to migrate into the interior of cells and adhere to proteins, particularly tubulin [45]. Inside the cell, the molecular rotor causes fluorescence emission from non-membrane components, violating the assumption of negligible background light that underlies Eq. (3). A new derivative featuring the membrane-compatible farnesyl group [24] dramatically improves membrane localization. It will be the subject of future studies to examine cellular microviscosity distribution with FCVJ by means of microscopic imaging. Application in cells will necessitate a different staining approach. FCVJ can be brought in contact with the cell membrane either when bound to proteins, particularly albumin [24,39], or a pre-stained liposome or micelle suspension can be brought in contact with the cells, causing the stained liposomes to fuse with the cell. One of the challenges with this method is to achieve consistent staining concentration. If this challenge cannot be met, examinations are restricted to changes within the same cell.

In two selected experiments, an excellent match between viscosity changes observed through FCVJ fluorescence intensity

and observed through the established method FRAP. While FRAP serves as the gold-standard method, the use of molecular rotors facilitates and accelerates the measurement of membrane viscosity changes, while maintaining high precision.

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