<http://www.diva-portal.org>

Postprint

This is the accepted version of a paper published in *Analytical Chemistry*. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the original published paper (version of record):

Chmyrov, V., Spielmann, T., Hevekerl, H., Widengren, J. (2015)

Trans-Cis isomerization of lipophilic dyes probing membrane microviscosity in biological membranes and in live cells.

Analytical Chemistry, 87(11): 5690-5697

<http://dx.doi.org/10.1021/acs.analchem.5b00863>

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:

<http://urn.kb.se/resolve?urn=urn:nbn:se:kth:diva-170226>

***Trans-cis* isomerization of lipophilic dyes probes membrane microviscosity in biological membranes and in live cells.**

Volodymyr Chmyrov, Thiemo Spielmann, Heike Hevekerl, Jerker Widengren

Experimental Biomolecular Physics, Department of Applied Physics, Royal Institute of Technology, Stockholm, Sweden.

Corresponding Author

Jerker Widengren: jerker@biomolphysics.kth.se.

ABSTRACT Membrane environment and fluidity can modulate the dynamics and interactions of membrane proteins, and can thereby strongly influence the function of cells and organisms in general. In this work, we demonstrate that *trans-cis* isomerization of lipophilic dyes is a useful parameter to monitor packaging and fluidity of biomembranes. Fluorescence fluctuations, generated by *trans-cis* isomerization of the thiocarbocyanine dye Merocyanine 540 (MC540) was first analyzed by Fluorescence Correlation Spectroscopy (FCS) in different alcohol solutions. Similar isomerization kinetics could then also be monitored of MC540 in lipid vesicles, and the influence of lipid polarity, membrane curvature and cholesterol content was investigated. While no influence of membrane curvature and lipid polarity could be observed, a clear decrease in the isomerization rates could be observed with increasing cholesterol contents in the vesicle membranes. Finally, procedures to spatially map photo-induced and thermal isomerization rates on live cells by transient state (TRAST) imaging were established. Based on these procedures, MC540 isomerization was studied on live MCF7 cells, and TRAST images of the cells at different temperatures were found to reliably detect differences in the isomerization parameters. Our studies indicate that *trans-cis* isomerization is a useful parameter for probing membrane dynamics, and that the TRAST imaging technique can provide spatial maps of photo-induced isomerization as well as both photo-induced and thermal back-isomerization, resolving differences in local membrane micro-viscosity in live cells.

INTRODUCTION

Physical properties of biological membranes, including degree of lipid packing, viscosity, stiffness and phase, play an important role in regulating interactions and activities of membrane protein receptors. These properties also influence the role of the membranes in providing catalytic surfaces, anchoring sites and electro-chemical barriers and thus have a major impact on cell function in general. Such properties have also been found to be part of the pathogenesis, or otherwise correlate with a wide range of different diseases. For instance, cell membrane fluidities have been found to be increased in cancer cells, and have even been found to correlate with worse prognoses^{1,2,3}. Lipid status and membrane dynamics is altered in inflammatory diseases⁴, and seem to play a significant role in the pathogenesis of Alzheimer's disease and of other age-related diseases, including cardiovascular diseases, diabetes and obesity^{5,6}.

To characterize and further understand the physical properties of biological membranes, how they influence fundamental processes in cells, and their role in different diseased states, several biophysical methods have been developed, based on NMR, EPR, IR, Raman or fluorescence spectroscopy techniques⁷. Fluorescence techniques offer high sensitivity, fast readouts and the use of low concentrations of probes, thereby minimizing perturbing effects on the membranes. They also offer high specificity with multiple readout parameters, and there exist a broad range of available probes reflecting different biomembrane properties. In combination, this makes fluorescence readouts of biomembrane dynamics well implementable for quantitative cellular imaging⁸ and high throughput readouts, including analyses by flow cytometry^{9,10,11}. Based on different fluorescence readout modalities and probes, occupying different locations in the membranes, different physical properties of membranes can be sensed. Fluorescence anisotropy measurements, using lipophilic dyes such as diphenylhexatriene (DPH)

provide information about rotational mobility of the dyes, reflecting membrane fluidity and order. Polarity-sensitive membrane probes, e.g. 6-dodecanoyl-2-dimethylamino naphthalene (Laurdan), can change their fluorescence emission spectra depending on the order of the immediate membrane environment¹². Membrane fluidity can also be assessed via formation of excimers, and the ratio of the spectrally separated emission from excimers and monomers, of certain lipophilic dyes, e.g. pyrene, which is dependent on the translational diffusion rate of the dyes. Finally, for microscopic arrangements membrane dynamics can be assessed by fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS). In general, the ability of fluorescent membrane probes to resolve different physical properties makes it interesting to combine them and their readouts for complementary information^{13,14}. This also motivates additional fluorescence readouts for membrane dynamic studies to be considered, representing additional complementary information.

Merocyanine 540 (MC540) is an anionic lipophilic polymethine dye, which binds superficially to the outer leaflets of membranes^{15,16}, and which is sensitive to subtle differences in lipid packing¹⁷. When the lipids in a membrane are loosely packed, MC540 is oriented parallel to the membrane surface, and immersed among the phospholipid chains¹⁴. With increased lipid packing, the dye takes an orientation perpendicular to the surface and superficial to the phospholipid chains. Its emission maximum is shifted from approx. 590 nm to approx. 620 nm and its fluorescence quantum yield drops. The spectral differences observed in these two states of MC540 can be used as readout of inter-lipid spacing, and to quantify co-existing liquid-crystalline and gel states of membranes.

The photophysical properties of MC540 and derivatives thereof have been investigated both when in organic solvents¹⁸⁻²⁰ and in small unilamellar vesicles (SUVs)¹⁶. As a polymethine dye, MC540 can undergo *trans-cis* isomerization, which can be monitored by FCS in a relatively straightforward manner²¹. From FCS solution measurements, on the dye Cy5²¹, as well as on MC540 in ethanol²², the isomerization rates have been found to well reflect local viscosity and temperature. This suggests that *trans-cis* isomerization dynamics possibly could be added to the list of fluorescence-based readout parameters useful for probing viscosity and temperature effects in biomembranes. However, most established technologies to monitor these states (e.g. transient absorption spectroscopy) are complicated and difficult to apply to a broad range of samples and have therefore been exploited to a very limited extent for biomolecular dynamics and interaction studies. By FCS, transitions to and from long-lived non- or weakly fluorescent states can be conveniently followed via fluctuations in the fluorescence intensity from a low number of dyes at a time. FCS-based monitoring of these states thus combines high detection sensitivity by the fluorescence readout, with high environmental sensitivity, given by the long lifetimes of the transient states. However, FCS measurements require fluorescent molecules with high brightness and in low concentrations, and highly sensitive detectors with high time resolution. This puts a limit to

the application range and throughput of FCS measurements, and makes parallel readouts and imaging difficult. To overcome these limitations, we have introduced an approach, so-called transient state (TRAST) imaging^{23,24}, where the fluorescent sample is subject to a modulated excitation. Depending on the excitation pulse train characteristics (e.g. pulse duration, separation and height) long-lived photo-induced transient states (e.g. triplet, photo-isomerized and photo-ionized states) of the fluorophores in the sample will be populated to different extents. Upon transient state population build-up in the sample, the fluorescence intensity will drop. By systematically varying the excitation pulse train characteristics and registering how the time-averaged fluorescence intensity changes the population kinetics of the transient states can then be retrieved. As with FCS, the TRAST technique also combines the high detection sensitivity of the fluorescence readout with the high environmental sensitivity of the dark transient states, but does not rely on a high fluorescent brightness of the molecules studied, on a high time resolution, or on a certain concentration of the sample. This makes TRAST imaging a broadly applicable approach that allows transient states, including photo-isomerized states of polymethine dyes, to be imaged by a standard CCD camera on live cells. This makes it also interesting to further consider the use of *trans-cis* isomerization as a biomembrane readout parameter.

The purpose of this work was to experimentally demonstrate the usefulness of *trans-cis* isomerization as a parameter to monitor packaging and viscosity of biomembranes. The *trans-cis* isomerization of MC540 was first studied by FCS in different alcohol solutions. It was then shown that the isomerization kinetics of MC540 could also be monitored together with SUVs, and the dependence of lipid polarity, membrane curvature and cholesterol content was investigated. Finally, MC540 isomerization was studied on live MCF7 cells by TRAST imaging, and isomerization images of the cells at different temperatures were recorded. The results demonstrate that *trans-cis* isomerization indeed can provide a useful parameter for probing membrane dynamics and that the TRAST imaging technique makes it possible to image and resolve differences in membrane microviscosity in live cells.

THEORY

Electronic state model. The photodynamic model used for MC540 has been previously presented^{21,22} and is further described in the SI. Under the conditions relevant for this study, the electronic state model of MC540 (figure S1B) can be reduced to a two-state model (figure S1C), containing a fluorescent *trans* form *N* and a non-fluorescent *cis* form *P*:

The effective rates of isomerization (k'_{ISO}) from *N* to *P* and back-isomerization (k'_{BISO}) from *P* to *N* will be the same as those from the excited state levels of *N* and *P* (k_{ISO} and k_{BISO}), except for a scaling factor correspond-

ing to the fractions of dyes in the N and P forms that are in their excited singlet states (N_1 and P_1):

$$k'_{ISO} = k_{ISO} \frac{k_{01}^{(N)}}{k_{01}^{(N)} + k_{10}^{(N)}} = k_{ISO} \frac{\sigma_N I_{exc}}{\sigma_N I_{exc} + k_{10}^{(N)}} \quad (2)$$

$$\begin{aligned} k'_{BISO} &= k_{BISO} \frac{k_{01}^{(P)}}{k_{01}^{(P)} + k_{10}^{(P)}} + k_{PN0} \\ &= k_{BISO} \frac{\sigma_P I_{exc}}{\sigma_P I_{exc} + k_{10}^{(P)}} + k_{PN0} \\ &= \{k_{10}^{(P)} \gg \sigma_P I_{exc}\} = \sigma_{BISO} I_{exc} + k_{PN0} \end{aligned} \quad (3)$$

Here, $k_{01}^{(X)}$, with $X = N$ or P , denote the excitation rate from the ground singlet electronic state X_0 to the first excited singlet state X_1 and $k_{10}^{(X)}$ is the deexcitation rate from X_1 to X_0 . I_{exc} is the excitation intensity, σ_N and σ_P are the excitation cross sections of the ground singlet states of N and P (N_0 and P_0), and $\sigma_{BISO} = \frac{k_{BISO}}{k_{10}^{(P)}} \sigma_P$ denotes the effective cross section for back-isomerization of the *cis* state.

Fluorescence Correlation Spectroscopy (FCS).

Given the basis of the FCS analyses, as outlined in the SI, with detected fluorescence intensity fluctuations generated from translational diffusion and from isomerization and back-isomerization of MC540 the time dependent part of the correlation function takes the form^{21, 22}:

$$\begin{aligned} G(\tau) &= \frac{1}{N_m} \left[1 + \frac{\tau}{\tau_D} \right]^{-1} \times \\ &\times \left[1 + \left(\frac{\omega_0}{\omega_z} \right)^2 \frac{\tau}{\tau_D} \right]^{-\frac{1}{2}} \left[1 + \frac{P_{eq}}{1 - P_{eq}} \exp(-\tau/\tau_{ISO}) \right] \end{aligned} \quad (4)$$

Here ω_0 and ω_z are the distances from the center of the laser beam focus in the radial and axial direction respectively at which the collected fluorescence intensity has dropped by a factor of $1/e^2$ compared to its peak value. N_m is the mean number of fluorescent molecules within the detection volume. τ_D is the characteristic diffusion time of the fluorescent molecules, given by the diffusion coefficient D as $\tau_D = \omega_0^2/4D$. P_{eq} is the time and space averaged fraction of fluorophores within the detection volume being in a non-fluorescent *cis* photoisomer form, and τ_{ISO} is the relaxation time related to the *trans-cis* isomerization process. Approximating the excitation irradiance distribution within the detection volume to be uniform²¹⁻²³, P_{eq} and τ_{ISO} of Equation 4 can be written as:

$$P_{eq} = \frac{k'_{ISO}}{k'_{ISO} + k'_{BISO}} \quad (5)$$

$$\tau_{ISO} = (k'_{ISO} + k'_{BISO})^{-1} \quad (6)$$

Transient state (TRAST) imaging. For a MC540 dye molecule excited by a rectangular laser pulse of height I_{exc} and duration t_p , the averaged fluorescence intensity emitted from the molecule within the duration of the excitation pulse is given by:

$$\langle F \rangle(t_p) = \frac{1}{t_p} \int_0^{t_p} k_{10}^{(N)} \Phi_f \frac{\sigma_N I_{exc}}{\sigma_N I_{exc} + k_{10}^{(N)}} N(t) dt \quad (7)$$

In a TRAST measurement, applying low duty cycle excitation pulse trains with different t_p , the isomerization parameters of MC540 can then in principle be determined from the dependence of $\langle F \rangle(t_p)$, as has been

shown for several other long-lived, photo-induced transient states^{23, 25, 26}. For MC540, this t_p -dependence is determined by the time dependence of the probability of the fluorophore to be in a *trans* state $N(t)$, which can be described by the model of equation 1, as further outlined in the SI. Following Equation 7, the population of the singlet excited *trans* state is given by:

$$N_1(t) = \frac{\sigma_N I_{exc}}{\sigma_N I_{exc} + k_{10}^{(N)}} N(t) \quad (8)$$

and for an excitation pulse train with pulse period T_p and with N_p number of pulses, the average fluorescence response from a fluorophore over the camera exposure time $t_{exp} = N_p T_p$, can be written as:

$$\begin{aligned} \langle F \rangle_{t_{exp}}(t_p) &= \frac{1}{t_{exp}} \int_0^{t_{exp}} \Phi_f k_{10}^{(N)} N_1(t) dt \\ &= \Phi_f \frac{\sigma_N I_{exc} k_{10}^{(N)}}{\sigma_N I_{exc} + k_{10}^{(N)}} \frac{1}{N_p T_p} \sum_{i=0}^{N_p-1} \int_{iT_p}^{iT_p+t_p} N(t) dt \end{aligned} \quad (9)$$

with ϕ_f denoting the fluorescence quantum yield of MC540. Combining Equation 9 and Equation S17 (which expresses the average population of the *trans* state within the excitation pulses of the same excitation pulse train, denoted $\langle N \rangle_{t_{exp}}(t_p)$), we get:

$$\begin{aligned} \langle F \rangle_{t_{exp}}(t_p) &= \eta \frac{\sigma_N I_{exc} k_{10}^{(N)}}{\sigma_N I_{exc} + k_{10}^{(N)}} \frac{\Phi_f}{t_p} \\ &\times \left[\left(\frac{k'_{BISO}}{-\lambda_2} + \frac{1}{\lambda_2} \langle P_{init} \rangle \right) (1 - \exp(\lambda_2 t)) + \frac{k'_{BISO}}{\lambda_2} t_p \right] \end{aligned} \quad (10)$$

Here, $\langle P_{init} \rangle$ denotes the average occupancy of the *cis* states at the onset of the excitation pulses within the pulse train (Equation S20). $-\lambda_2 = k'_{ISO} + k'_{BISO}$ denotes the effective relaxation rate of the *trans-cis* isomerization (Equation S9), and $\eta = t_p/T_p$ is the excitation duty cycle. The derivation of Equation 10 makes no assumptions regarding relaxation of photo-induced states in the time between the pulses ($T_p - t_p$), and thus holds for arbitrary pulse durations, as recently published²⁸. In a TRAST experiment, based on wide-field excitation, the average fluorescence intensity over an exposure time of t_{exp} detected by a pixel (α, β) on the camera then reads:

$$\bar{F}_D(\alpha, \beta, t_p) = c(\alpha, \beta) q \langle F \rangle_{t_{exp}}(x_\alpha, y_\beta, t_p) \quad (11)$$

Here, $c(\alpha, \beta)$ denotes the MC540 dye concentration in the volume centered at (x_α, y_β) , imaged by a pixel located at (α, β) . $\langle F \rangle_{t_{exp}}(x_\alpha, y_\beta, t_p)$ signifies the average fluorescence intensity emitted from a MC540 dye molecule within the volume centered at (x_α, y_β) , generated by an effective irradiance given by:

$$\begin{aligned} I'_{exc}(x_\alpha, y_\beta) &= \frac{\iiint I_{exc}(\vec{r}) F_S^{01}(I_{exc}(\vec{r}))}{\iiint F_S^{01}(I_{exc}(\vec{r}))} \\ &\cdot \frac{CEF(x-x_\alpha, y-y_\beta, z) dx dy dz}{CEF(x-x_\alpha, y-y_\beta, z) dx dy dz} \end{aligned} \quad (12)$$

For wide-field microscopy, $I_{exc}(\vec{r})$ is assumed to be Gaussian distributed in the radial directions of the excitation laser beam. The fluorescence $\bar{F}_S^{01}$ takes only into account the singlet state equilibration and is determined by:

$$\bar{F}_S^{01}(I_{exc}(\vec{r})) = \Phi_f \frac{\sigma_N I_{exc}(\vec{r}) k_{10}^{(N)}}{\sigma_N I_{exc}(\vec{r}) + k_{10}^{(N)}} \quad (13)$$

The collection efficiency function (CEF) for one pixel of the camera is approximated by the convolution between

the transmission function of the pinhole projected to the focal plane, and the point spread function (PSF)²⁹.

MATERIALS AND METHODS

Sample preparation. Details regarding preparations of solutions, liposomes and calibration cover slides, as well as cell culturing conditions are given in the SI.

FCS, TRAST and Spectrofluorometer measurements.

The major features of the FCS setup used has previously been described,³⁰⁻³² (see SI for details). The setup used for the TRAST experiments is based on a previously described instrument^{26, 27} with some modifications, providing either a confocal detection or a wide-field microscopy read-out for the live cell measurements (see SI). Excitation and emission spectra were measured by a spectrofluorometer (Fluoromax, Horiba Jobin Yvon). See SI for further details.

TRAST data analysis. The position and extension of the wide-field beam in the sample were estimated using fluorescence images of new, unbleached regions of cover glasses labeled with Atto-Thio 12. These images were taken at sufficiently short pulse widths to avoid dark states build up. The extension of the excitation beam was then estimated to $1/e^2$ radii of 85 μm and 118 μm by fitting the acquired image to a 2D Gaussian distribution. By mapping the position of the excitation beam onto the camera and using the determined radii, the absorption cross-section of the dye and the measured power after the objective could be determined, and an effective excitation rate could be assigned to each pixel on the camera (Equations 12-13).^{23, 26} Similarly, for confocal measurements the beam widths were calculated by applying a 2D Gaussian distribution fit to the each image of the sample, yielding $1/e^2$ radii of 0.9 μm /0.5 μm and 1.6 μm /1.15 μm in ethanol and in the vesicle solution respectively.

The non-zero extinction factor of the AOM (approximately 0.1%) was accounted for by subtracting an image recorded with the AOM turned off from the other images. To reduce processing work and to improve the fluorescence signal statistics, the resolution of the cell images was lowered using the default bicubic interpolation algorithm in Matlab, increasing the pixel sizes from 250 to 500 nm. Then a threshold of 10 to 20% of the maximum fluorescence intensity was applied to get rid of the background pixels and to fit only the data in pixels belonging to cells.

To estimate the decrease in fluorescence signal due to photobleaching during the TRAST measurements reference images were intermittently acquired at the shortest excitation pulse width. For every pixel and every applied irradiance, these curves were fitted to a single or a double exponential and the matrix containing the fitted decay parameters was then used to correct the acquired TRAST signal.

In a last step, a matrix pixel-wise Marquart-Levenberg fit is applied to the TRAST curves according to Equation 11, and with the effective excitation rate assigned to

each pixel. For each pixel, five TRAST curves at different I_{exc} were acquired, and subsequently fitted globally to Equation 11, having k_{ISO} and σ_{BISO} as free parameters, and considering the effective excitation rates by use of Equations 12-13.

RESULTS AND DISCUSSION

Isomerization kinetics of MC540 in ethanol and in lipid vesicles.

To investigate to what extent isomerization could be detected for MC540 in lipid membranes, and how well the isomerization parameters reflect the dynamic and mechanical properties of the membrane, FCS measurements of MC540 in ethanol and in lipid vesicles were performed. Liposomes made of DOPC lipids were used as a simple model, offering stable and reproducible membrane properties. The recorded FCS curves were fitted to Equation 4, with N_m , τ_D , P_{eq} and τ_{ISO} as free parameters. Clear differences could be observed between the ethanol and liposome measurements (Figure 1A). τ_D increased by almost two orders of magnitude, from 79 μs for MC540 in ethanol solution, up to 7 ms for the liposome measurements. An almost equally large difference could be observed for τ_{ISO} . For FCS curves recorded from MC540 in ethanol P_{eq} was found to be higher (around 56%) than when recorded from the liposome samples (around 30%) (Figures 1A, 1C and 1D). The observed changes in the isomerization kinetics can be attributed to the different environmental conditions. When attached to a lipid membrane, MC540 is likely to display slower isomerization rates, due to the presumably larger viscous drag experienced. This is well in line with previous FCS solution studies, where τ_{ISO} for the dye Cy5 was found to increase in proportion to the viscosity of the solution²¹. In the FCS curves recorded from the liposome samples one extra exponential relaxation process in the time range of 100 ns could be noticed, reasonably well in agreement with the theoretically expected rotational correlation times ($\tau_{rot} = 100$ ns, from the Debye expression) of the vesicles used (100 nm diameter). In the fitting of the FCS curves from the vesicle measurements, the rotation time was therefore hereinafter set to be a constant and kept at 100 ns.

To further analyze differences in the isomerization kinetics between the samples, a set of FCS curves was recorded with different excitation irradiances (power series), with I_{exc} varying from 5 up to 107 kW/cm^2 . P_{eq} and $1/\tau_{ISO}$, determined from the ethanol and the vesicle samples by fitting the individual FCS curves to Equation 4 are plotted in Figures 1C and 1D, and were in turn subject to fitting by use of Equation 5 and 6 (including Equations 1 and 2), with k_{ISO} and σ_{BISO} as free parameters, and with σ_N and $k_{10}^{(N)}$ fixed to $4.05 \times 10^{-16} \text{ cm}^2$ and $2 \times 10^9 \text{ s}^{-1}$ respectively. By this procedure, the kinetic rate constants were determined to $k_{ISO} = 46.99 \times 10^6 \text{ s}^{-1}$, $\sigma_{BISO} = 0.21 \times 10^{-16} \text{ cm}^2$ for the liposomes, and to $k_{ISO} = 172.68 \times 10^6 \text{ s}^{-1}$, $\sigma_{BISO} = 0.27 \times 10^{-16} \text{ cm}^2$ for the measurements in solution. Isomerization dynamics of MC540 in DOPC vesicles were also investigated by confocal TRAST, yielding rates in agreement with the FCS measurements ($k_{ISO} = 44.09 \times 10^6 \text{ s}^{-1}$, $\sigma_{BISO} = 0.19 \times 10^{-16} \text{ cm}^2$). Overall, for both the ethanol and the vesicle sam-

ples P_{eq} remained constant and $1/\tau_{ISO}$ showed a linear dependence on I_{exc} (Figure 1), well in accordance with the I_{exc} -dependence expected from the electronic state model (Figure S1B and S1C, Equation 1).

Dependence of isomerization kinetics on lipid charge, membrane curvature and fluidity. To investigate what membrane properties could possibly influence the recorded isomerization parameters, FCS measurements were performed with MC540 attached to liposomes of different sizes, with different surface charge, and with the membrane fluidity in the vesicle membranes changed by mixing different amounts of cholesterol into the vesicles.

Figure 1: **(A)** Normalized (N_m set to unity) FCS curves recorded from MC540 dissolved in ethanol (solid circles) and when attached to liposomes (empty circles). The FCS curves were fitted to Equation 4, and residuals are shown below **(B)**. Significant differences in diffusion times, τ_D , between the samples are noticeable (for the curves shown, 79 μ s and 7 ms, respectively). **(C)**: Experimental and fitted fraction of photo-isomerized MC540 P_{eq} and isomerization relaxation rates $1/\tau_{ISO}$ from FCS measurements in ethanol. **(D)**: corresponding data from DOPC liposome measurements.

To test the influence of the membrane surface charge, P_{eq} and τ_{ISO} , recorded by FCS from MC540 in zwitterionic DOPC vesicles and in negatively charged DOPG vesicles of the same size (100 nm) were compared. No differences were detected (data not shown). The isomerization of the MC540 fluorophores was also investigated by FCS in solvents with different polarities (methanol, ethanol, butanol and propanol). As for the DOPC vs. DOPG comparison, no difference in the isomerization

kinetics was detected (data not shown). Based on these results it can be concluded that the isomerization of MC540 fluorophore is at least in these environments independent of the charge and polarity of the surrounding medium.

Next, effects of the membrane curvature on isomerization kinetics were investigated, by performing FCS measurements on MC540-labelled liposomes with different diameters (30 nm, 50 nm, 100nm and 200 nm). Also for these measurements, no difference in the isomerization dynamics was detected, giving no indications that the isomerization process would depend of membrane curvature. From previous studies on lipid vesicles, it has been reported that the brightness of MC540 is inversely proportional to the vesicle size (i.e. that the fluorophore is brighter when attached to a smaller vesicle, with more loosely packed outer leaflet lipids)¹⁷. In our FCS experiments a slight change in the molecular brightness of the fluorophores could also be detected (20% decrease in brightness for the 100 nm vesicles compared to the 30 nm ones). However, since no differences in the isomerization fractions, P_{eq} , nor in the relaxation times, τ_{ISO} were revealed, the isomerization process is not likely to be the reason behind these changes in the fluorophore brightness.

Figure 2: Normalized FCS curves recorded from MC540 associated to DOPC liposomes with different cholesterol concentrations. Insert: k_{ISO} and σ_{BISO} versus cholesterol concentration in the liposomes.

To investigate the consequences of membrane fluidity changes, MC540-labelled liposomes (made from DOPC lipids) were prepared with different cholesterol concentrations and the isomerization kinetics of MC540 were

subsequently studied by FCS. With higher cholesterol contents in the lipid membranes (Figure 2) a clear increase in τ_{ISO} was found, indicating a lowering of the membrane mobility. These observations are well in line with earlier reports, stating a lowered membrane fluidity upon addition of cholesterol into vesicle membranes (POPC, fluid phase),³³ as well as into membranes of live cells (U373-M6 glioblastoma cells).² For the DOPC/cholesterol liposomes, and in a similar fashion as described above (Figure 1), FCS curves were recorded at different I_{exc} (from 5 to 107 kW/cm²), fitted to Equation 4, and from the determined parameters P_{eq} and τ_{ISO} , the isomerization parameters k_{ISO} and σ_{BISO} were then determined from Equation 5 and 6. With increasing cholesterol concentrations, both k_{ISO} and σ_{BISO} were found to decrease. The isomerization parameters showed a good sensitivity to changes in fluidity, with differences of 10% in cholesterol contents clearly distinguishable (inset, Figure 2). From FCS measurements performed at different I_{exc} , and for cholesterol concentrations ranging from 0% to 50%, k_{ISO} was found to decrease from $\sim 46.99 \times 10^6 \text{ s}^{-1}$ to $\sim 28.23 \times 10^6 \text{ s}^{-1}$ and σ_{BISO} from $\sim 0.233 \times 10^{-16} \text{ cm}^2$ to $\sim 0.162 \times 10^{-16} \text{ cm}^2$.

In contrast, from spectrofluorometer measurements only a very minor shift could be observed in the excitation and emission spectra of MC540 labeled DOPC vesicles with different cholesterol contents. For vesicles with 50% compared to 0% cholesterol contents, the excitation and emission spectra were only red-shifted by about 2 nm ($\lambda_{exc.max}$ from 566 nm to 568 nm, $\lambda_{em.max}$ from 584 nm to 586 nm). The way lipophilic dyes associate to lipid membranes can indeed vary from one dye to another, and can also depend on the membrane properties. Still, it is worth noting that our observations for MC540 is in line with recent atomistic simulations of the expected isomerization rates of the lipophilic carbocyanine dye Dil, which indicate that the isomerization rates directly reflect the lipid headgroup viscosity.³⁴ Our data further indicate that the isomerization characteristics of MC540 are influenced by other membrane features than its fluorescence intensity, or its excitation and emission spectra, and that the MC540 isomerization kinetics are much more sensitive to the presence of cholesterol than are the MC540 spectral changes.

Isomerization measurements in live cells with TRAST. Following the solution and liposome studies by FCS, we next investigated to what extent isomerization dynamics could also be monitored and imaged in live cells. MCF7 cells were labeled with MC540 and TRAST measurements were performed as described above (and in the SI), with pixelwise recording of average fluorescence intensities, generated upon wide-field excitation by pulse trains with different pulse durations (from 1 μs to 1 ms). The recorded average fluorescence intensities versus the width of the excitation pulses (TRAST curves) were then analyzed based on Equation 10 and 11.

TRAST curves were acquired in each pixel, and at five different excitation irradiances (linearly spaced between 45 and 240 W/cm²). A typical set of TRAST curves is shown in Figure 3A, averaged over a region of interest (ROI) consisting of several cells, and showing the typical

decrease of the fluorescence intensity at increasing pulse widths due to the build-up of a non-fluorescent, photo-induced, transient state. For increasing excitation irradiances the decay times of the TRAST curves are shifted to shorter times, while the amplitude of the relaxation process remains approximately constant. These findings are well in line with the excitation irradiance dependence of the isomerization parameters P_{eq} and τ_{ISO} , as recorded by FCS in this work (Figures 1C,D) and in previous studies.^{21,22} Taken together, this allowed us to associate the dark state observed in the TRAST measurements with the isomerization of MC540.

The recorded TRAST were found to be well described by a single exponential term according to Equation 10 and 11, indicating the presence of only a single dark state. Compared to the FCS measurements on lipid vesicles, with isomerization relaxation times in the range of 1-10 μs (Figure 2), the TRAST curves showed relaxation times at much slower time scales (50-200 μs , Figure 3). This is due to the much lower excitation irradiances applied in wide-field TRAST, compared to those used in the FCS measurements. In contrast to e.g. singlet-triplet state transitions^{23,28}, the transitions between the *trans* and *cis* states are predominantly excitation induced in both directions, and prominent *cis*-state populations can be generated also at quite moderate excitation intensities. With TRAST imaging, isomerization kinetics can thus in general be studied at considerably lower irradiances than triplet state population kinetics. At excitation wavelengths in the green range (514 nm), light exposures in live cell measurements have been recommended not to exceed 100 J/cm².³⁵ In this work, the light exposure on the cells during the TRAST measurements are well below this level (19 J/cm², including recording of TRAST curves at all five excitation irradiances applied), and indicates that also triplet state imaging by TRAST is feasible at tolerable light exposures for the cells.

Figure 3: **(A)** Set of five TRAST curves, recorded at different irradiances with peak irradiances between 45 and 240 W/cm² (increased irradiances in direction of the arrow). The curves show the normalized fluorescence (averaged over a cell at 37 °C as the one shown in Figure 4, third row), versus the applied pulse width, t_p , and a global fit (based on Equations 10 and 11) applied to the same data. In the experiments, the duty cycle was kept constant at 0.5%. **(B)** TRAST curves averaged over different cells measured at different temperatures. For each temperature five TRAST curves were recorded as outlined above. However, for clarity only the recorded TRAST curves at the mid irradiance (peak pulse irradiance of 150 W/cm²) is shown, even though the fit (lines) comprises the set of five TRAST curves measured at different irradiances as in (A).

The set of recorded TRAST curves averaged over the whole cells (as in Figures 3A, 3B) can be well described by a model based on Equation 10 and 11 with k_{ISO} , σ_{BISO} and k_{PNO} as free parameters using an averaged I'_{exc} . In this fit, as well as in all following fits $k_{10}^{(N)}$ and σ_N are fixed to 2000 μs^{-1} and 5.3×10^{-16} cm² ($\lambda = 561$ nm) respectively. Next, the set of five TRAST curves generated in each pixel were fitted independently with an I'_{exc} estimated for each pixel using Equation 12. In these fits k_{ISO} and σ_{ISO} were left as free parameters whereas k_{PNO} was fixed to the value determined for the set fluorescence of curves averaged over the same cell or set of cells. Typi-

cal k_{ISO} and σ_{ISO} images obtained from the fit are shown in Figure 4 (third and fourth column). In these images, the sensitivity of the isomerization kinetics to membrane fluidity was tested by varying the temperature in three steps (24 °C, 30 °C, and 37 °C). For each temperature, five ROIs were imaged as described before (a set of TRAST curves is obtained for each pixel, by applying excitation pulse trains with varying t_p at five different excitation irradiances). In Figure 3B, mean TRAST curves are shown measured at the three different temperatures and averaged over three ROIs. From the curves, the relaxation amplitudes, proportional to the build-up of the *cis* photo-isomer, can be seen to increase with temperature. Moreover, the characteristic relaxation time is shifted to faster timescales with increasing temperatures. This behavior corresponds to an increase of k_{ISO} with increased temperatures.

In Figure 4, three ROIs are presented measured at 24 °C, 30 °C and 37 °C. For each ROI, phase contrast, fluorescence, k_{ISO} and σ_{BISO} images are presented. As expected from the averaged curves in Figure 3B a net increase of k_{ISO} is visible for higher temperatures, whereas σ_{BISO} seems not to be influenced so much by increased temperatures. This is further confirmed by the 2-D histograms in the fifth column of the Figure 4, each comprising the pixels of 4 cellular images measured at a defined temperature. They show that the average k_{ISO} increases from $\sim 60 \times 10^6$ s⁻¹ to $\sim 80 \times 10^6$ s⁻¹ for temperatures increasing from 24°C to 37°C, while σ_{BISO} remains essentially constant (0.3×10^{-16} cm²). The resulting k_{ISO} and σ_{BISO} rates are overall in reasonable agreement with the values obtained by FCS (inset Figure 2). Since membrane fluidity is expected to increase with temperature, these results are in agreement with the FCS experiments (Figure 2) and show similar dependences to the changes in fluidity.

In Figure 4 (fifth column), a strong correlation between the parameters k_{ISO} and σ_{BISO} can be seen in the individual histograms as the 2D-distributions show an inclination. This observed correlation between k_{ISO} and σ_{BISO} is likely not an artifact from the fitting procedure indicating that the two parameters are not determined independently, but rather comes from the fact that both parameters are similarly influenced by the same aspects of the membrane micro-environment. On the other hand, the slope of the inclination decreases with increasing temperatures, and the temperature effects are mainly seen for k_{ISO} , indicating that the parameters k_{ISO} and σ_{BISO} are also not completely correlated and do not reflect exactly the same micro-environmental information.

Finally, as discussed above, for each data analysis k_{PNO} was initially determined using the mean fluorescence averaged over each ROI. The obtained mean values and standard deviations recorded from the 4 different ROIs (as also in Figure 4) were found for each temperature to be: $k_{PNO} = (0.41 \pm 0.03) \text{ ms}^{-1}$ (24 °C), $k_{PNO} = (0.64 \pm 0.05) \text{ ms}^{-1}$ (30 °C) and $k_{PNO} = (0.89 \pm 0.08) \text{ ms}^{-1}$ (37 °C). Hence, a clear temperature dependence is also visible for the thermal deactivation rate k_{PNO} . With higher temperatures a slight reduction in fluorescence intensity was observed in the fluorescence images (20-30% reduction). This could be due to a reduction in

ϕ_f at higher temperatures or to fluorophore dissociation from the membrane over time since the measurements at 37 °C were taken last. However, since the TRAST method considers only relative fluorescence changes due to variation of t_p , the obtained k_{ISO} and σ_{BISO} images are not biased by this decrease in fluorescence brightness, nor are the determined k_{PN0} rates.

Figure 4: First row: MCF7 cells at 24 °C, second row: 30 °C, third row: 37 °C. First column – phase contrast images, second – fluorescence images, third and fourth columns k_{ISO} and σ_{BISO} images obtained by TRAST (see main text for details), fifth column – 2-D histograms of k_{ISO} and σ_{BISO} and each containing the pixels from 4 different ROIs (5092, 6402 and 8614 pixels respectively). A fit of the distributions yields the following mean values and standard deviations: (B₁): $k_{ISO} = (58.0 \pm 9.9) \mu s^{-1}$, $\sigma_{BISO} = (0.31 \pm 0.05) \times 10^{-16} cm^2$, (B₂): $k_{ISO} = (72.5 \pm 9.7) \mu s^{-1}$, $\sigma_{BISO} = (0.32 \pm 0.04) \times 10^{-16} cm^2$ and (B₃): $k_{ISO} = (84.2 \pm 15.0) \mu s^{-1}$, $\sigma_{BISO} = (0.32 \pm 0.05) \times 10^{-16} cm^2$.

CONCLUSIONS

Trans-cis isomerization in polymethine dyes can reflect local viscosity not only in solution, but also in biological membranes. By two different techniques (FCS and TRAST) and two major environmental parameters (membrane cholesterol content and temperature), known to influence the membrane fluidity, the isomerization of MC540 was confirmed to enable sensitive monitoring of the membrane state. By the procedures established in this work, isomerization parameters can be accurately and reliably measured in live cells, and thereby these parameters can offer a complement to, and also extend the capabilities of established fluorescence readouts of membrane fluidity and microenvironment. Given the large relevance of biological membranes and their influence on fundamental cellular processes, the use of the

isomerization process as a source of information in biomembrane research, and the procedures for their readout as established in this work, are likely to find further use both in fundamental cell biology and for diagnostic applications.

ACKNOWLEDGMENT

This work was supported by means from EU FP7 (FLUODIAMON 201 837), the Swedish Research Council (VR-NT 2012-3045), and the Knut and Alice Wallenberg Foundation (KAW 2011.0218). T.S. was supported by the National Research Fund, Luxembourg. The authors would like to thank Andriy Chmyrov and Jonas Mücksch for discussions, and Cobolt AB, Solna, Swe-

den, for kindly lending out the 561nm laser used in the TRAST measurements

ASSOCIATED CONTENT

Supporting Information

Theoretical background (electronic state model, FCS and TRAST), materials and methods (sample preparation, FCS setup, TRAST setup and spectrofluorometer measurements). Detailed derivation of the fitting model. This material is available free of charge via the Internet at <http://pubs.acs.org>.

REFERENCES

- (1) Kaur, J.; Sanyal, S. N. *Mol Cell Biochem* **2010**, 341, (1-2), 99-108.
- (2) Sok, M.; Sentjurs, M.; Schara, M.; Stare, J.; Rott, T. *Ann Thorac Surg* **2002**, 73, (5), 1567-71.
- (3) Boutin, C.; Roche, Y.; Millot, C.; Deturche, R.; Royer, P.; Manfait, M.; Plain, J. M.; Jeannesson, P.; Millot, J. M.; Jaffiol, R. *J Biomed Opt* **2009**, 14, (3), 034030.
- (4) Tai, W. Y.; Yang, Y. C.; Lin, H. J.; Huang, C. P.; Cheng, Y. L.; Chen, M. F.; Yen, H. L.; Liao, I. *J Phys Chem B* **2010**, 114, (47), 15642-9.
- (5) Florent-Bechard, S.; Desbene, C.; Garcia, P.; Allouche, A.; Youssef, I.; Escanye, M. C.; Koziel, V.; Hanse, M.; Malaplate-Armand, C.; Stenger, C.; Kriem, B.; Yen-Potin, F. T.; Olivier, J. L.; Pillot, T.; Oster, T. *Biochimie* **2009**, 91, (6), 804-9.
- (6) Chauhan, N. B. *J Lipid Res* **2003**, 44, (11), 2019-29.
- (7) Melo, E.; Martins, J. *Biophys Chem* **2006**, 123, (2-3), 77-94.
- (8) Weber, P.; Wagner, M.; Schneckenburger, H. *J Biomed Opt* **2010**, 15, (4), 046017.
- (9) Masuda, M.; Kuriki, H.; Komiyama, Y.; Nishikado, H.; Egawa, H.; Murata, K. *J Immunol Methods* **1987**, 96, (2), 225-31.
- (10) Gillan, L.; Evans, G.; Maxwell, W. M. *Theriology* **2005**, 63, (2), 445-57.
- (11) McCarthy, D. A.; Macey, M. G.; Allen, P. D. *Exp Hematol* **2008**, 36, (8), 909-21.
- (12) Parasassi, T.; De Stasio, G.; d'Ubaldo, A.; Gratton, E. *Biophys J* **1990**, 57, (6), 1179-86.
- (13) Stott, B. M.; Vu, M. P.; McLemore, C. O.; Lund, M. S.; Gibbons, E.; Brueske, T. J.; Wilson-Ashworth, H. A.; Bell, J. D. *J Lipid Res* **2008**, 49, (6), 1202-15.
- (14) Wilson-Ashworth, H. A.; Bahm, Q.; Erickson, J.; Shinkle, A.; Vu, M. P.; Woodbury, D.; Bell, J. D. *Biophys J* **2006**, 91, (11), 4091-101.
- (15) Lelkes, P. I.; Miller, I. R. *J Membr Biol* **1980**, 52, (1), 1-15.
- (16) Onganer, Y.; Quitevis, E. L. *Biochim Biophys Acta* **1994**, 1192, (1), 27-34.
- (17) Stillwell, W.; Wassall, S. R.; Dumauld, A. C.; Ehringer, W. D.; Browning, C. W.; Janski, L. J. *Biochim Biophys Acta* **1993**, 1146, (1), 136-44.
- (18) Benniston, A. C.; Harriman, A.; McAvoy, C. *Journal of the Chemical Society-Faraday Transactions* **1998**, 94, (4), 519-525.
- (19) Redmond, R. W.; Kochevar, I. E.; Krieg, M.; Smith, G.; McGimpsey, W. G. *Journal of Physical Chemistry A* **1997**, 101, (15), 2773-2777.
- (20) Benniston, A. C.; Harriman, A.; Gulliya, K. S. *Journal of the Chemical Society-Faraday Transactions* **1994**, 90, (7), 953-961.
- (21) Widengren, J.; Schwill, P. *Journal of Physical Chemistry A* **2000**, 104, (27), 6416-6428.
- (22) Widengren, J.; Seidel, C. A. M. *Physical Chemistry Chemical Physics* **2000**, 2, (15), 3435-3441.
- (23) Sanden, T.; Persson, G.; Thyberg, P.; Blom, H.; Widengren, J. *Analytical Chemistry* **2007**, 79, (9), 3330-3341.
- (24) Sanden, T.; Persson, G.; Widengren, J. *Analytical Chemistry* **2008**, 80, (24), 9589-9596.
- (25) Widengren, J. *Journal of the Royal Society Interface* **2010**, 7, (49), 1135-1144.
- (26) Spielmann, T.; Blom, H.; Geissbuehler, M.; Lasser, T.; Widengren, J. *Journal of Physical Chemistry B* **2010**, 114, (11), 4035-4046.
- (27) Spielmann, T.; Xu, L.; Gad, A. K. B.; Johansson, S.; Widengren, J. *FEBS Journal*, **2014**, 218, (5), 1317-1332.

(28) Mücksch, J., Spielmann, T., Sismakis, E., Widengren, J. Journal of Biophotonics, available online DOI: 10.1002/jbio.201400015.

(29) Widengren, J.; Mets, Ü.; Rigler, R. Journal of Physical Chemistry **1995**, 99, (36), 13368-13379.

(30) Stromqvist, J.; Chmyrov, A.; Johansson, S.; Andersson, A.; Mäler, L.; Widengren, J. Biophysical Journal **2010**, 99, (11), 3821-3830.

(31) Chmyrov, A.; Sanden, T.; Widengren, J. Journal of Physical Chemistry B **2010**, 114, (34), 11282-11291.

(32) Rigler, R.; Mets, Ü.; Widengren, J.; Kask, P. European Biophysics Journal with Biophysics Letters **1993**, 22, (3), 169-175.

(33) Arora, A.; Raghuraman, H.; Chattopadhyay, A. Biochemical and Biophysical Research Communications **2004**, 318, (4), 920-926.

(34) Muddana, H. S.; Gullapalli, R. R.; Manias, E.; Butler, P. J. Phys Chem Chem Phys **2011**, 13, (4), 1368-78.

(35) Wagner, M.; Weber, P.; Bruns, T.; Strauss, W. S. L.; Wittig, R.; Schneckenburger, H. International Journal of Molecular Sciences **2010**, 11, (3), 956-966.

Trans-cis isomerization of lipophilic dyes probes membrane microviscosity in biological membranes and in live cells.

Volodymyr Chmyrov, Thiemo Spielmann, Heike Hevekerl, Jerker Widengren¹

Experimental Biomolecular Physics, Department of Applied Physics,

Royal Institute of Technology, Stockholm, Sweden.

Table of contents

• Theory	S-2
○ Electronic state model	S-2
○ Fluorescence Correlation Spectroscopy (FCS)	S-3
○ Transient State (TRAST) Imaging	S-4
• Materials and Methods	S-5
○ Sample preparation	S-5
○ FCS setup	S-6
○ TRAST setup	S-6
○ Spectrofluorometer measurements	S-7
• References	S-7

¹ Corresponding author: Jerker Widengren,
Email: jerker@biomolphysics.kth.se

THEORY

Electronic state model.

The photodynamic model used for the dye Merocyanine 540 (MC540) is shown in Figure S1. At equilibrium, with no excitation, MC540 adopts an all-*trans* conformation. Upon excitation, one of the double bounds in the polymethine chain of the dye can undergo a π -twist around the bond axis and the molecule switches into a non-fluorescent *cis* isomer. Likewise, when in a *cis* state, excitation-driven back-isomerization can take the MC540 molecule back to an all-*trans* state. These transitions, and the states involved are defined in Figure 1B, where also a more detailed description of the model is given.

Figure S1: (A): Chemical structure and conformations of the *trans* and *cis* isomers of Merocyanine 540. (B): Generally used model for *trans-cis* isomerization of cyanine dyes, where the horizontal direction corresponds to torsion angle around a double bond of the polymethine chain of MC540, and the vertical direction corresponds to Energy. N_0 and N_1 denote the ground singlet and the first excited singlet state of the all-*trans* form. $k_{01}^{(n)} = \sigma_N I_{exc}$ and $k_{10}^{(n)}$ are the rates of excitation from N_0 to N_1 , and deactivation from N_1 to N_0 . P_0 and P_1 , and $k_{01}^{(p)} = \sigma_P I_{exc}$ and $k_{10}^{(p)}$ are the corresponding states and rates of the mono-*cis* form. *Perp* is the intermediary twisted excited state at half the rotation angle. It is formed from N_1 or P_1 at rates k_{NPerp} and k_{PPerp} , respectively, and deactivated in the picosecond to nanosecond range to either N_0 or P_0 . P_0 can thermally relax to the ground state of the all-*trans* form, N_0 , with a rate denoted by k_{PN0} . This transition typically takes place in the millisecond time range. (C): Simplified photophysical model for cyanine dyes, with for our FCS and TRAST measurements relevant simplifications, supported by observations made for other cyanine dyes^{1, 2}. Transitions to and from the partially twisted intermediate state, *Perp*, as well as transitions between the singlet states within the *trans* and *cis* forms are disregarded since they take place on a time scale much faster than the switching between the *trans* and *cis* isomers. The *Perp* state is typically deactivated in the picosecond to nanosecond

time scale to either N_0 or P_0 , and the deactivation times of N_1 and P_1 are in the nanosecond time scale. These transitions will therefore not be resolved on the time scale of the FCS and TRAST measurements. Since most *cis*-isomers of thiocarbocyanine dyes are believed not to fluoresce at room temperature, and upon excitation to mainly be deactivated through internal conversion the fluorescence brightness of the *cis* photo-isomer can be neglected. Triplet state formation from either N_1 or P_1 is also in general very low for thiocarbocyanine dyes, and can therefore be disregarded in the model, and for the measurement conditions used in this study.

Based on time-averaging of the fast transitions between the states of Figure S1B and disregarding any triplet state formation in the dyes, the kinetic schedule of Figure S1B can be simplified to the two-state model of Figure S1C, containing a fluorescent *trans* form N (N_0 and N_1) and a non-fluorescent *cis* form P (P_0 and P_1). The effective transition rate constants from N and P will be the same as those from the excited state levels of N and P, except for a scaling factor corresponding to the fractions of dyes in the N and P forms that are in their excited singlet states:

$$k'_{ISO} = k_{ISO} \frac{k_{01}^{(N)}}{k_{01}^{(N)} + k_{10}^{(N)}} = k_{ISO} \frac{\sigma_N I_{exc}}{\sigma_N I_{exc} + k_{10}^{(N)}} \quad (S1)$$

$$\begin{aligned} k'_{BISO} &= k_{BISO} \frac{k_{01}^{(P)}}{k_{01}^{(P)} + k_{10}^{(P)}} + k_{PN0} \\ &= k_{BISO} \frac{\sigma_P I_{exc}}{\sigma_P I_{exc} + k_{10}^{(P)}} + k_{PN0} \\ &= \left\{ k_{10}^{(P)} \gg \sigma_P I_{exc} \right\} = \sigma_{BISO} I_{exc} + k_{PN0} \end{aligned} \quad (S2)$$

where I_{exc} is the excitation intensity, σ_N and σ_P are the excitation cross sections of the N_0 and P_0 states, and $\sigma_{BISO} = \frac{k_{BISO}}{k_{10}^{(P)}} \sigma_P$ denotes the effective cross section for back-isomerization of the *cis* state.

Fluorescence Correlation Spectroscopy (FCS).

Based on a confocal, epi-illuminated instrument, the detected fluorescence rate in the FCS measurements is given by:

$$F(t) = \int CEF(\vec{r}) c(\vec{r}, t) k_{10}^{(N)} q \Phi_f N_1(\vec{r}, t) dV \quad (S3)$$

Here, $CEF(\vec{r})$ is the collection efficiency function of the confocal microscope setup, $c(\vec{r}, t)$ denotes the concentration of fluorophores. q the quantum efficiency of the detectors and the attenuation of the fluorescence in the passage from the sample volume to the detector areas, Φ_f the fluorescence quantum yield of the fluorophore, and $N_1(\vec{r}, t)$ the fraction of the fluorophores that are in their excited singlet *trans* states. The fluorescence fluctuations are caused by changes in $N_1(\vec{r}, t)$ and by changes in $c(\vec{r}, t)$, caused by translational motion of the fluorophores into and out of the detection volume. If fluorescence fluctuations arise from translational diffusion, assuming a 3-dimensional Gaussian distribution of the detected fluorescence, and from fluorescence blinking originating from transitions between a fluorescent *trans* isomer, N , and a non-fluorescent *cis* isomer, P , as modeled in Figure S1C, the time dependent part of the correlation function takes the form^{1, 2}:

$$G(\tau) = \frac{1}{N_m} \left[1 + \frac{\tau}{\tau_D} \right]^{-1} \times \left[1 + \left(\frac{\omega_0}{\omega_z} \right)^2 \frac{\tau}{\tau_D} \right]^{-\frac{1}{2}} \left[1 + \frac{P_{eq}}{1 - P_{eq}} \exp(-\tau/\tau_{ISO}) \right] \quad (S4)$$

Here ω_0 and ω_z are the distances from the center of the laser beam focus in the radial and axial direction respectively at which the collected fluorescence intensity has dropped by a factor of $1/e^2$ compared to its peak value. N_m is the mean number of fluorescent molecules within the detection volume. τ_D is the characteristic diffusion time of the fluorescent molecules, given by the diffusion coefficient D as $\tau_D = \omega_0^2/4D$. P_{eq} is the time and space averaged fraction of fluorophores within the detection volume being in a non-fluorescent *cis* photoisomer form, and τ_{ISO} is the relaxation time related to the

trans-cis isomerization process. Approximating the excitation irradiance distribution within the detection volume to be uniform¹⁻³, P_{eq} and τ_{ISO} of Equation 4 can be written as:

$$P_{eq} = \frac{k'_{ISO}}{k'_{ISO} + k'_{BISO}} \quad (S5)$$

$$\tau_{ISO} = (k'_{ISO} + k'_{BISO})^{-1} \quad (S6)$$

Transient state (TRAST) imaging

For a MC540 molecule subject to a train of rectangular excitation laser pulses of height I_{exc} and duration t_p , the time dependence of the fluorophore to be in a *trans* state, $N(t)$, can be described by the model of Figure S1C. Following the model of Figure S1C, $N(t)$ is obtained via a system of linear differential equations which in matrix form can be written as:

$$\frac{d}{dt} \vec{S}(t) = \mathbf{M} \vec{S}(t) \quad (S7)$$

$$\vec{S}(t) = \begin{pmatrix} N(t) \\ P(t) \end{pmatrix} \quad \mathbf{M} = \begin{pmatrix} -k'_{ISO} & k'_{BISO} \\ k'_{ISO} & -k'_{BISO} \end{pmatrix} \quad (S8)$$

Here, $\vec{S}$ is the population vector of the *trans*, N , and *cis*, P , states and $\mathbf{M}$ is a coupling matrix.

The eigenvalues of $\mathbf{M}$ are given by:

$$\begin{cases} \lambda_1 = 0 \\ \lambda_2 = -k'_{ISO} - k'_{BISO} \end{cases} \quad (S9)$$

and the corresponding pair of orthogonal eigenvectors by:

$$\vec{v}_1 = \begin{pmatrix} k'_{BISO} \\ k'_{ISO} \end{pmatrix}, \quad \vec{v}_2 = \begin{pmatrix} -1 \\ 1 \end{pmatrix} \quad (S10)$$

Ideally in a TRAST experiment, applying a train of rectangular excitation pulses with a low duty cycle, the non-fluorescent transient state(s) should within the time between the pulses ($T_p - t_p$, with T_p denoting the period time) relax back to the ground state of the fluorescent form of the dye. However, the isomerization to and from P are mainly excitation-driven (Figure S1B). In the absence of excitation there is only a slow thermal deactivation rate, k_{PNO} , of P, typically in the range of 0,1-1 ms⁻¹. The *cis* state does therefore not necessarily relax back completely to the ground *trans* state during the off-periods, i.e. in between the excitation pulses of the pulse trains used in the TRAST experiments.

For a correct representation of the fluorescence response to a rectangular pulse train of arbitrary duty cycle, the solution to Equation S1 that can be written as:

$$\vec{S}(t) = \sum c_i \vec{v}_i \exp(\lambda_i t) = \mathbf{Q} \exp(\mathbf{\Lambda} t) \vec{C} \quad (S11)$$

which could then be rewritten in the form of a propagator:

$$\vec{S}(t) = \mathbf{Q} \exp(\mathbf{\Lambda} t) \mathbf{Q}^{-1} \vec{S}(t_0) = \mathbf{U}(t, t_0) \vec{S}(t_0) \quad (S12)$$

Where $\vec{v}_i$ and λ_i are eigenvectors and eigenvalues respectively. c_i represents a set of constants, obtained from the initial conditions to Equation S1. $\mathbf{\Lambda}_{ii} = \lambda_i$ is the diagonal eigenvalue matrix, $\mathbf{Q}$ is the eigenvector matrix having as its i -th column the eigenvector $\vec{v}_i$. $\vec{C}$ is a vector containing the constants c_i . The matrix $\mathbf{U}(t, t_0)$ describes how the system evolves over time from an initial state $\vec{S}(t_0)$.

$$\mathbf{Q} = \begin{pmatrix} k'_{BISO} & -1 \\ k'_{ISO} & 1 \end{pmatrix} \quad \mathbf{Q}^{-1} = \frac{1}{k'_{ISO} + k'_{BISO}} \begin{pmatrix} 1 & -1 \\ -k'_{ISO} & k'_{BISO} \end{pmatrix} \quad (S13)$$

Taking into account that $\forall t, N(t) + P(t) = 1$, arbitrary initial conditions $\vec{S}(t_0)$ can be written as:

$$\vec{S}(t_0) = \begin{pmatrix} 1-P(t_0) \\ P(t_0) \end{pmatrix} \quad (S14)$$

The evolution of the solution of the initial system (Equation S7) can then be calculated as a function of an arbitrary initial population of the *cis*-state $P(t_0)$:

$$\begin{pmatrix} N(t) \\ P(t) \end{pmatrix} = \begin{cases} \frac{k'_{BISO}}{k'_{BISO} + k'_{ISO}} + \left(\frac{k'_{BISO}}{k'_{BISO} + k'_{ISO}} - P(t_0) \right) \exp(\lambda_2 t) \\ \frac{k'_{ISO}}{k'_{BISO} + k'_{ISO}} - \left(\frac{k'_{ISO}}{k'_{BISO} + k'_{ISO}} - P(t_0) \right) \exp(\lambda_2 t) \end{cases} \quad (S15)$$

By use of Equation S15, and for a fluorophore subject to a rectangular excitation pulse train (with N_p number of pulses, pulse period T_p , and duration of pulses t_p), and resetting the time t in Equation 9 to zero after each period of the excitation pulse train, the average population of $N = N_0 + N_1$ within the excitation pulses can be obtained from the last sum over the integrals part of Equation S15 as:

$$\begin{aligned} \langle N \rangle_{t_{exp}}(t_p) &= \frac{1}{N_p t_p} \sum_{i=0}^{N_p-1} \int_{iT_p}^{iT_p+t_p} N(t) dt = \\ &= \int_0^{t_p} \frac{1}{N_p t_p} \sum_{j=1}^{N_p} \left[\frac{k'_{BISO}}{-\lambda_2} + \left(\frac{k'_{ISO}}{-\lambda_2} - P_j(0) \right) \exp(\lambda_2 t) \right] dt \end{aligned} \quad (S16),$$

where $P_j(0)$ is the *cis* state population at the onset of the j 'th pulse. Since all rates and eigenvalues are constant over $t \in [0, t_p]$ integration yields:

$$\langle N \rangle_{t_{exp}}(t_p) = \frac{1}{t_p} \left[\left(\frac{k'_{BISO}}{-\lambda_2} + \frac{1}{\lambda_2} \langle P_{init} \rangle \right) (1 - \exp(\lambda_2 t_p)) + \frac{k'_{BISO}}{\lambda_2} t_p \right] \quad (S17)$$

Here, the time averaged population of the *trans* state for a pulse train of pulse width t_p , denoted $\langle N \rangle_{t_{exp}}(t_p)$, involves the average initial occupancy of the *cis* state at the onset of all the pulses within the pulse train: $\langle P_{init} \rangle = \frac{1}{N_p} \sum_{j=1}^{N_p} (P_j(0))$.

In order to calculate $\langle P_{init} \rangle$ a propagator was defined for the time during which the pulse is on $\mathbf{U}^{on}(t_p, 0)$ and one for which the excitation is off $\mathbf{U}^{off}(T_p, t_p)$. For $\mathbf{U}^{off}(T_p, t_p)$, $k'_{ISO} = 0$ and $k'_{BISO} = k_{pN0}$ since by definition I_{exc} is equal to zero during the off-times. The evolution of the populations at time $t = T_p$, after one full pulse period starting at $t_0 = 0$ is then given by:

$$\vec{S}(T_p) = \mathbf{U}^{off}(T_p, t_p) \mathbf{U}^{on}(t_p, 0) \vec{S}(t_0) \quad (S18)$$

Similarly, the population status of N and P at $t = t_{exp} = N_p T_p$, after a full pulse train consisting of N_p pulses, is found by applying the N_p times the propagator.

$$\vec{S}(N_p T_p) = \left(\mathbf{U}^{off}(T_p, t_p) \mathbf{U}^{on}(t_p, 0) \right)^{N_p} \vec{S}(t_0) \quad (S19)$$

Finally, the propagators can be used in order to calculate $\langle P_{init} \rangle$, as well as the average population of N and P at the onset of an excitation pulse within the pulse train:

$$\begin{pmatrix} \langle N_{init} \rangle \\ \langle P_{init} \rangle \end{pmatrix} = \frac{1}{N_p} \sum_{j=0}^{N_p-1} \begin{pmatrix} \langle N_{j(0)} \rangle \\ \langle P_{j(0)} \rangle \end{pmatrix} = \frac{1}{N_p} \sum_{j=0}^{N_p-1} [\mathbf{U}^{off}(T_p, t_p) \mathbf{U}^{on}(t_p, 0)]^j \vec{S}(t_0) \quad (S20)$$

MATERIALS AND METHODS

Sample preparation.

A 2 mM stock solution of Merocyanine 540 (MC540, Sigma-Aldrich) was prepared in ethanol. DOPC, DOPG and cholesterol (Avanti Polar Lipids) were obtained dissolved in Chloroform (10mg/mL) or as a powder. DOPC and DOPG liposomes were prepared as previously described⁴, in a 0.2 mM, pH 7.4 KCl solution, and cholesterol in varying percentages

was included when stated. For the liposome formation, a mini-extruder (Avanti) with membrane filters containing 30, 50, 100 and 200 nm diameter pores was used. For the FCS measurements, the fluorophore-to-lipid ratios were kept quite low (1:1.000.000) for all vesicles sizes to ensure that each liposome had no more than one fluorophore. For the spectrofluorometer measurements the fluorophore-to-lipid ratio was higher (1:30.000). For confocal TRAST measurements, where isomerization kinetics is independent on labeling efficiency, the fluorophore-to-lipid ratio were set relatively high (1:1.000).

For the live cell measurements MCF7 cells were grown adherent in an eight well plate for three to four days. Then cells were washed once with PBS buffer and 300 μ L of 5 μ M MC540 dissolved in serum free PBS was added to the wells. Cells were incubated for 20 minutes at 37°C, and were then washed three times with a growth medium (containing FBS), with 5 min incubation in between each washing step.

In order to estimate the dimension of the wide field beam, cover slides were labeled with ATTO-Thio 12 (Atto-tec, Siegen, Germany) using Bovine serum albumin (BSA) as a linker. A droplet of BSA solution was deposited on the cover glass slides and incubated for 20 minutes at room temperature. After three washing steps with deionized water, a droplet of PBS solution containing 10 μ M of Atto-Thio 12 was applied to the slides. In this step the dye bound to the protein by amine binding. After incubating for at least 10 minutes, the sample was washed 3 times with PBS and kept in this medium until experiment.

FCS setup.

The major features of the setup used has previously been described.⁵⁻⁷ Briefly, for excitation the 568nm line of a krypton-argon laser (Melles Griot 643-RYB-A02, with excitation filter Z568/10, Chroma Technology Corp.) was reflected by a dichroic beam splitter (FF576/661-Di01-18-D, Semrock Inc.) and then focused on the sample by a water-immersion objective (Zeiss, 63x/1.2 Plan-Neofluar, 160 mm tube length, cover glass corrected). In ethanol solutions the laser beam was focused down to radius ($1/e^2$) of 0.35 μ m, while in the vesicle solution the focal beam radius was 0,7 μ m. Fluorescence was collected by the same objective, passed through a 50 μ m pinhole in the image plane, and focused onto two avalanche photodiodes (APDs) (SPCM-AQR-14, Perkin-Elmer Optoelectronics) in a beam splitting arrangement. Two APDs were used in order to eliminate all influence of the inherent noise, and afterpulsing effects of the detectors. To discriminate background from Raman and Rayleigh scattered laser light, a band-pass filter (HQ640/115M, Chroma Technology Corp.) was inserted in front of each APD. The signal from the two APDs was analyzed by a PC-based correlator (ALV-5000, ALV GmbH). The laser power was controlled by a laser power controller (BEOC LPC) and measured before the objective by a laser power meter (PM-100, Thor Labs), subsequently corrected for the transmission through the objective.

TRAST setup.

The setup used for the TRAST experiments is based on a previously described instrument, with some modifications.⁸ The beam of a CW diode-pumped solid state laser (561 nm, 15 mW to 75 mW, Cobolt Jive, Cobolt AB) was focused by a lens (focal distance of 80 mm) into the aperture of an acousto-optic modulator (AOM) (MQ 180-AO, 25-VIS, AA OptoElectronic). After passing the AOM, the beam was collimated by a second lens (focal distance of 300 mm), and then directed either to the back port of a microscope stand (Zeiss 135 TV) or to a home built confocal microscope. For the live-cell measurements at the microscope stand (Zeiss 135 TV), a collimated wide-field beam was generated using a 40 $\times$ 0.75 Ph2 objective from Zeiss. The microscope stand was equipped with a dichroic mirror (Di01-R561, Semrock) and a fluorescence filter (HQ 610/75, AHF), as well as with an incubation system (Chamlide IC, Live Cell Instrument, Seoul,

Korea) for maintaining constant sample temperatures (variable from room temperature 23 °C to 45 °C). An automatic three gas mixing system (FC-7, LCI) was used to set the partial pressures of O₂, CO₂ and N₂ gas in the incubator. During cell experiments, O₂ was kept constant at about 7%, and CO₂ at 5% in order to keep the pH of the culturing medium constant.

An electron multiplying CCD camera (658×498 pixels, sized 10 × 10 μm², photon quantum yield of 50%, Luca, Andor Technology), mounted on the camera mount of the microscope stand, was used for imaging the fluorescence emitted from the sample.

For liposome measurements in a confocal arrangement the laser beam was after passage through the AOM directed to another dichroic mirror (Di01-R561, Semrock) and focused on sample by a water immersion objective (Plan-NEOFLUAR 40x/1.2 Zeiss). The fluorescence was collected by the same objective, passed through a fluorescence filter (HQ 610/75, AHF) and directed to the CCD camera where each pixel was considered as a pinhole.

Custom software was used for controlling signal detection and further signal processing. The camera was controlled in Matlab via USB using a driver based on the MCD Software Development Kit from Andor Technology, and the laser via serial port commands. The modulation scheme of the AOM was set via a PCI 6602 card (National Instruments) connected to the AOM controller (AA Opto Electronic).

Spectrofluorometer measurements.

Excitation and emission spectra of MC540 in aqueous solution and of MC540-labeled DOPC/Cholesterol liposome solutions were measured by a spectrofluorometer (Fluoromax, Horiba Jobin Yvon). Excitation spectra within 500 to 600 nm were recorded at 640 nm emission, and emission spectra (540 to 650 nm) were recorded at 520 nm excitation.

REFERENCES

- (1) Widengren, J.; Schwille, P. *Journal of Physical Chemistry A* 2000, 104, (27), 6416-6428.
- (2) Widengren, J.; Seidel, C. A. M. *Physical Chemistry Chemical Physics* 2000, 2, (15), 3435-3441.
- (3) Sanden, T.; Persson, G.; Thyberg, P.; Blom, H.; Widengren, J. *Analytical Chemistry* 2007, 79, (9), 3330-3341.
- (4) Sanden, T.; Salomonsson, L.; Brzezinski, P.; Widengren, J. *Proceedings of the National Academy of Sciences of the United States of America* 2010, 107, (9), 4129-4134.
- (5) Stromqvist, J.; Chmyrov, A.; Johansson, S.; Andersson, A.; Maler, L.; Widengren, J. *Biophysical Journal* 2010, 99, (11), 3821-3830.
- (6) Chmyrov, A.; Sanden, T.; Widengren, J. *Journal of Physical Chemistry B* 2010, 114, (34), 11282-11291.
- (7) Rigler, R.; Mets, U.; Widengren, J.; Kask, P. *European Biophysics Journal with Biophysics Letters* 1993, 22, (3), 169-175.
- (8) Spielmann, T.; Blom, H.; Geissbuehler, M.; Lasser, T.; Widengren, J. *Journal of Physical Chemistry B* 2010, 114, (11), 4035-4046.