# Single-Molecule Anisotropy Imaging

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ABSTRACT A novel method, single-molecule anisotropy imaging, has been employed to simultaneously study lateral and rotational diffusion of fluorescence-labeled lipids on supported phospholipid membranes. In a fluid membrane composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, in which the rotational diffusion time is on the order of the excited-state lifetime of the fluorophore rhodamine, a rotational diffusion constant, $D_{rot} = 7 \times 10^7$ rad$^2$/s, was determined. The lateral diffusion constant, measured by direct analysis of single-molecule trajectories, was $D_{lat} = 3.5 \times 10^{-8}$ cm$^2$/s. As predicted from the free-volume model for diffusion, the results exhibit a significantly enhanced mobility on the nanosecond time scale. For membranes of DPPC lipids in the $L_\beta$ gel phase, the slow rotational mobility permitted the direct observation of the rotation of individual molecules characterized by $D_{rot} = 1.2$ rad$^2$/s. The latter data were evaluated by a mean square angular displacement analysis. The technique developed here should prove itself profitable for imaging of conformational motions of individual proteins on the time scale of milliseconds to seconds.

## INTRODUCTION

The dynamical behavior of various components of cellular membranes is a major determinant for the regulation of protein and cell function (Edidin, 1987). Lateral mobility in these membranes has typically been measured by fluorescence recovery techniques (fluorescence recovery after photobleaching, FRAP) (Axelrod et al., 1976; Tamm and Kalb, 1992), while the rotational behavior was studied by the polarization equivalent of photobleaching (pFRAP) (Tamm and Kalb, 1992; Velez and Axelrod, 1988; Timbs and Thompson, 1990, 1993), by fluorescence correlation spectroscopy (Aragon and Percora, 1975; Widengren et al., 1995), and by nuclear (Selig, 1977; Cullis and de Kruiff, 1976; Vaz et al., 1979; MacKay, 1981; Gosh, 1988) and electron spin resonance (Shin and Freed, 1989). Newer techniques, developed to study the lateral mobility of membrane constituents, have added a more detailed understanding of the processes that underlie membrane motion. Labeling of membrane components by either gold or latex beads (single-particle tracking) (Geerts et al., 1987; Quian et al., 1991; Lee et al., 1991; Anderson et al., 1992; Gosh and Webb, 1994) or by direct labeling of the molecule by a single fluorophore (single-molecule microscopy) (Schmidt et al., 1996) allowed for the study of their trajectories in real time. Single-particle tracking and single-molecule microscopy are superior to fluorescence photobleaching and fluorescence correlation spectroscopy because their spatial resolution can be as small as a few nanometers (Gelles et al., 1988). Despite the advantage of single-particle tracking, labeling with large tags like fluorescent beads does not allow one to determine rotational dynamics because the observed signal, either the fluorescence from a bead or the scattered light of a gold particle, is depolarized. In contrast, a quantum system like a single fluorophore has a well-defined transition dipole moment with respect to its structure and thus is suited for the study of rotational dynamics by optical means. This advantage has clearly been demonstrated in several examples for single-molecule rotational studies (Ha et al., 1996, 1998; Schütz et al., 1997a; Dickson et al., 1998) and fluorescence correlation spectroscopy studies (Widengren et al., 1995; Eigen and Rigler, 1994; Schaffer et al., 1999).

One goal in the study of biomembranes by single-molecule techniques is the simultaneous observation of the lateral and rotational mobilities of individual fluorescence-labeled membrane components. This has been achieved in the current study. By the introduction of a polarizing element into the optical detection path, it was possible to follow the lateral and the rotational trajectories of individual fluorescence-labeled lipid molecules on a phospholipid membrane by using a CCD camera system. Thus for the first time it has become possible to determine the lateral and rotational mobilities of a single molecule simultaneously. Our achievement opens up the possibility of observing, in real time, conformational motions of individual peptides and proteins indicative of structural changes accompanying biological function.

## EXPERIMENTAL PROCEDURES

Phospholipid membranes of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, Avanti) and DPPC (1, 2-dipalmitoyl-sn-glycero-3-phosphocholine, Avanti) were deposited at room temperature (22°C) on glass slides by Langmuir-Blodgett deposition as previously described (Schmidt et al., 1996) or by vesicle fusion (Kalb et al., 1992). Small unilamellar vesicles (SUVs) were prepared by sonicating a buffer solution (phosphate-buffered saline) containing 5 mg/ml phospholipid in a sonicator bath for 30

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min. The membranes were formed by deposition of 50 $\mu$l of a solution of SUVs onto cleaned glass substrates for several minutes. The glass slides were previously soaked in chromic acid for several hours and, after they were thoroughly rinsed in ultrapure water, they were dried in an Ar atmosphere. After the membranes were formed, the slides were gently flushed with buffer. The supported membranes, prepared by either technique, were subsequently placed in a 10-$\mu$m-thick cuvette and mounted on the microscope. On preparation of the vesicles the lipids were doped with small amounts ($10^{-9}$ mol/mol) of fluorescent tetramethylrhodamine-labeled (TMR-DPPE) (Molecular Probes; T-1391) or cyanine-labeled (Cy7-DPPE) (Kada, 1998) lipids. At this concentration the surface density of fluorescent molecules after fusion to the substrate was $<0.01$ molecules/$\mu$m$^2$. The samples showed no difference in photophysical or mobility behavior between the two methods of preparation.

The experimental arrangement for single-molecule imaging has been described in detail by Schmidt et al. (1995) (see also Fig. 1). Essentially, the samples were mounted on an inverted microscope equipped with a 100× objective (NA 1.3; Zeiss PlanNeofluar) and illuminated for 5–20 ms with either 514-nm light (tetramethylrhodamine) from an $Ar^+$ laser (C306; Coherent) or 695-nm light from a laser diode (TOLD9150, Toshiba, controlled by an ILX Lightwave controler, LDC-3744). The illumination intensity was set at 5 kW/cm$^2$ in all experiments. Control of the polarization of the excitation light was achieved by the introduction of a $\lambda/4$ wave plate and/or a polarizing beam splitter mounted on a rotation stage in the light path. For linear polarized light, the degree of polarization measured by means of a polarizing beam splitter at the position of the sample was better than 20:1. This slight depolarization is due partly to the use of a high-NA objective (Axelrod,

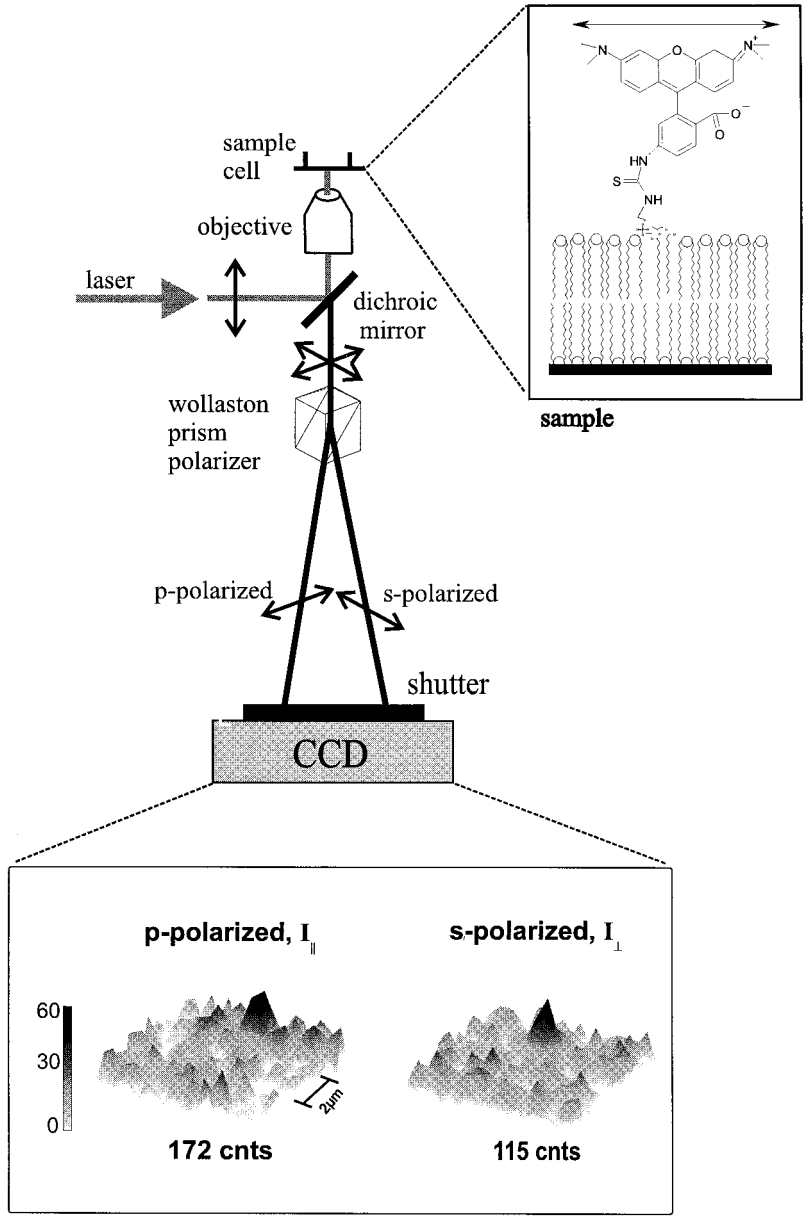


FIGURE 1 Experimental setup. A laser beam with selected polarization (see text) is reflected onto the sample. The sample, a supported phospholipid membrane, contains fluorescence-labeled lipids at low concentration. The molecular structure of the fluorescent molecule (TMR), with its absorption and emission axis marked above, is shown in the upper insert. The polarization of the fluorescence, collected through the objective, was separated by a Wollaston polarizer. The polarization-separated images were detected on two regions of a CCD camera. The lower image inset shows the signal from a single TMR-labeled lipid in a POPC membrane illuminated for 5 ms at an intensity of 5 kW/cm$^2$. Counts of 172 ± 5 and 115 ± 6 were detected in the p- and s-polarized channels, respectively.

1979). Any systematic error due to this slight degree of depolarization was covered by the intrinsic random errors (shot noise) and was therefore neglected. Appropriate filter combinations permitted the detection of single fluorophores in a membrane by a nitrogen-cooled CCD camera (AT360, Photometrics, equipped with a EEV1024 CCD chip) with a detection efficiency of >3%. The fluorescence images were virtually free of background signal and were taken consecutively with a delay between 15 and 150 ms.

In previous publications we have shown that this setup allows for the imaging of individual molecules with a time resolution of milliseconds and a lateral accuracy of 40 nm (Schmidt et al., 1995, 1996). In the current study, the aim was to simultaneously determine the degree of the fluorescence polarization and the lateral diffusion. To achieve this, a Wollaston prism polarizer (Zeta Optics) was placed into the microscope, splitting the p- and s-polarized light by an angle of 5° (see Fig. 1). p- and s-images were simultaneously projected onto the lower part of the camera (opposite the serial shift register) and read out by the same scheme as described in detail by Schmidt et al. (1995). Typical fluorescence images for TMR-labeled lipids are shown at the bottom of Fig. 1. The polarization characteristics of the dichroic beamsplitter, objectives, and filters were carefully determined and conceived for all intensity measurements. The correction factor, $g$, which describes the different detection efficiencies, $\eta$, in the p- and s-channels, was determined to be $g_{TMR} = \eta_s/\eta p = 0.915$ for TMR and $g_{Cy7} = 0.907$ for Cy7.

## RESULTS AND DISCUSSION

Fig. 2 exhibits typical results obtained for individual TMR-labeled lipid molecules in fluid phospholipid membranes (POPC). An area of $10 \times 10\ \mu m^2$ was illuminated for 10 ms, with the polarization of the excitation light set parallel to the polarization of the p-detection channel (Fig. 2 *A*). The resulting fluorescence intensity distributions in the p- and s-channels are shown (*left and right columns*, respectively). As seen in the figure, the fluorescence signal of an individual molecule (*black*) is clearly distinguishable from the background signal (*gray*). Consecutive illuminations at a delay of 30 ms allowed us to determine both the lateral trajectory and the direction of the polarization of individual molecules. The lateral mobility was a random motion characterized by a mean lateral diffusion constant, $D_{lat} = 3.5 \pm 0.4\ 10^{-8}$ cm$^2$/s, in registry with previous reports (Schütz et al., 1997b).

The image sequence in Fig. 2 *A* clearly suggests that the fluorescence intensity in the p-channel is higher than that in the s-channel. After correction with the polarization efficiency factor, $g_{TMR}$, the fluorescence signals in Fig. 2 *A* were determined to be $I_p = 285 \pm 17$ counts for the p-channel and $I_s = 130 \pm 12$ counts for the s-channel. From the latter values, a two-dimensional anisotropy, $r = (I_p - I_s)/(I_p + I_s)$, of $0.37 \pm 0.07$ was determined. The two-dimensional anisotropy has been taken here because it is known for fluorophores like rhodamines that they orient with their molecular plane parallel to the surface of a membrane (Axelrod, 1979; Lieberherr et al., 1987; Timbs and Thompson, 1993). From the definition of the anisotropy given above, the mean two-dimensional anisotropy on linear excitation, for a molecule rotating slowly with respect to its excited-state lifetime (a few nanoseconds) but fast with respect to the total time of observation (10 ms here), is $r = 0.5$. On the contrary, for a molecule that rotates fast with respect to its excited-state lifetime, the anisotropy is $r = 0$. As an additional control, measurements using excitation by

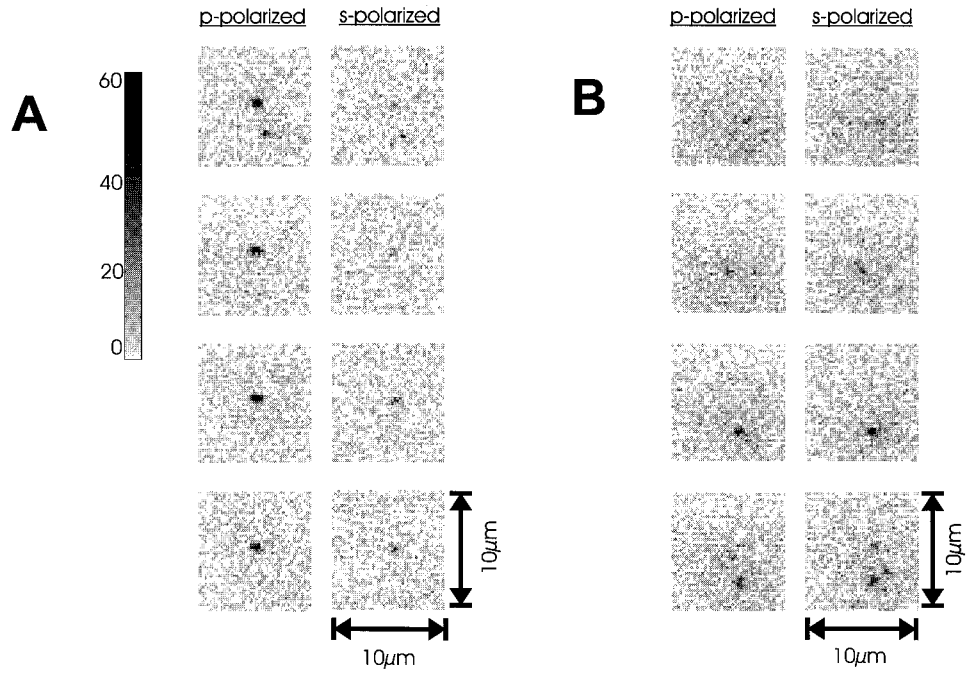


FIGURE 2 Consecutive images of a single TMR-labeled lipid molecule in a POPC membrane. The exposure time was 10 ms, the intensity was 5 kW/cm$^2$, and the delay between consecutive images was 30 ms. The images are scaled between zero (*white*) and 60 counts/pixel (*black*). (*A*) On linearly polarized excitation a mean intensity of $285 \pm 17$ counts and $130 \pm 12$ counts in the p- and s-polarized channels, respectively, was detected for the molecule shown. The anisotropy was determined to be $0.37 \pm 0.05$, corresponding to $D_{rot} = 0.4 \pm 0.2\ 10^8$ rad$^2$/s. (*B*) Control experiment using circularly polarized excitation. In this case, the p- and s-polarized mean intensities were $136 \pm 25$ counts and $149 \pm 22$ counts, respectively, resulting in an anisotropy of $r = -0.05 \pm 0.10$.

circularly polarized light were performed (Fig. 2 *B*). As expected, the fluorescence signals observed (149 ± 22 counts and 136 ± 25 counts for the p- and s-channels, respectively) are identical within the error bars, resulting in an anisotropy of $r < 0.05$.

Experiments as exemplified in Fig. 2 have been performed for rhodamine- and cyanine-labeled phospholipids in fluid membranes. The results are summarized in Fig. 3, *A* and *B*, as histograms from 204 and 257 (TMR and Cy7) individual molecules, respectively. With linear polarized excitation the distributions were characterized by $\langle r_{lin} \rangle = 0.30 \pm 0.01$ for TMR (weighted mean ± standard deviation of the mean; Bevington and Robinson, 1992) and $\langle r_{lin} \rangle = 0.46 \pm 0.05$ for Cy7. The widths of the distributions in $r$ of ~0.1 are fully explained by the shot-noise-limited intensity determination as corroborated by Monte Carlo simulations. Control experiments using circularly polarized light (Fig. 3, *A* and *B*, *dashed columns*, $\langle r_{circ} \rangle = -0.07 \pm 0.01$ and $0.10 \pm 0.01$, respectively), and light polarized 45° with respect to the Wollaston axis (data not shown, $\langle r_{45°} \rangle = -0.01 \pm 0.02$ for TMR) yielded values for the anisotropy that are close to zero, as would be predicted.

Monte Carlo simulations were performed for $10^6$ excitation-emission cycles of a rotating molecule at random start angles (equivalent to an ensemble average of randomly oriented molecules) to obtain quantitative values of the two-dimensional anisotropy. The fluorescence lifetime of the molecule was taken to be exponentially distributed with a mean of $\tau$. The molecular rotation about a central axis was described by a Gaussian distribution of rotation angles of mean 0 and width, $\sigma^2 = 2D_{rot}t$ (Chandrasekhar, 1943), characterized by a rotational diffusion constant, $D_{rot}$, and the time between absorption and emission of a photon, $t$. It was further assumed that the rotation could take place only about the membrane normal, i.e., in one dimension. This assumption is rationalized by former findings, which showed that >88% of such fluorophores align their transition dipole moments perpendicular to the membrane (Axelrod, 1979; Lieberherr et al., 1987; Timbs and Thompson, 1993). An initial decrease in the anisotropy due to a differ-

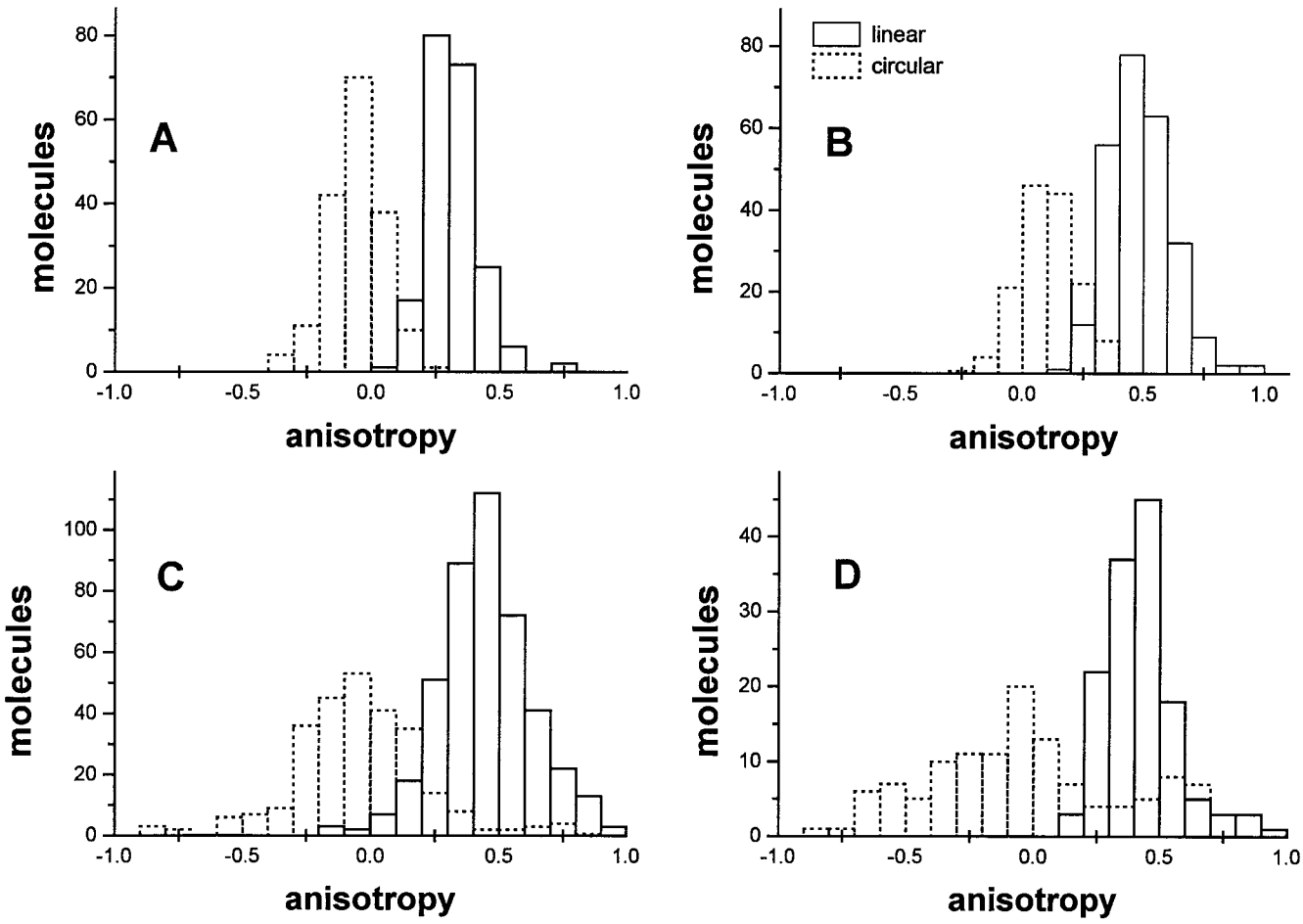


FIGURE 3 Histograms of the anisotropies determined from individual fluorescence-labeled lipid molecules. The solid and dotted lines indicate anisotropy measurements using linearly ($r_{lin}$) and circularly ($r_{circ}$) polarized excitation light, respectively. (*A*) TMR:POPE in POPC. $\langle r_{lin} \rangle = 0.30 \pm 0.01$ ($N = 204$ observations), and $\langle r_{circ} \rangle = -0.07 \pm 0.01$ ($N = 177$). (*B*) Cy7:POPE in POPC, $\langle r_{lin} \rangle = 0.46 \pm 0.05$ ($N = 257$), and $\langle r_{circ} \rangle = 0.10 \pm 0.01$ ($N = 146$). (*C*) TMR:POPE in DPPC, $\langle r_{lin} \rangle = 0.41 \pm 0.05$ ($N = 440$), and $\langle r_{circ} \rangle = -0.05 \pm 0.01$ ($N = 271$). (*D*) Cy7:POPE in DPPC, $\langle r_{lin} \rangle = 0.39 \pm 0.10$ ($N = 137$), and $\langle r_{circ} \rangle = -0.09 \pm 0.01$ ($N = 128$).

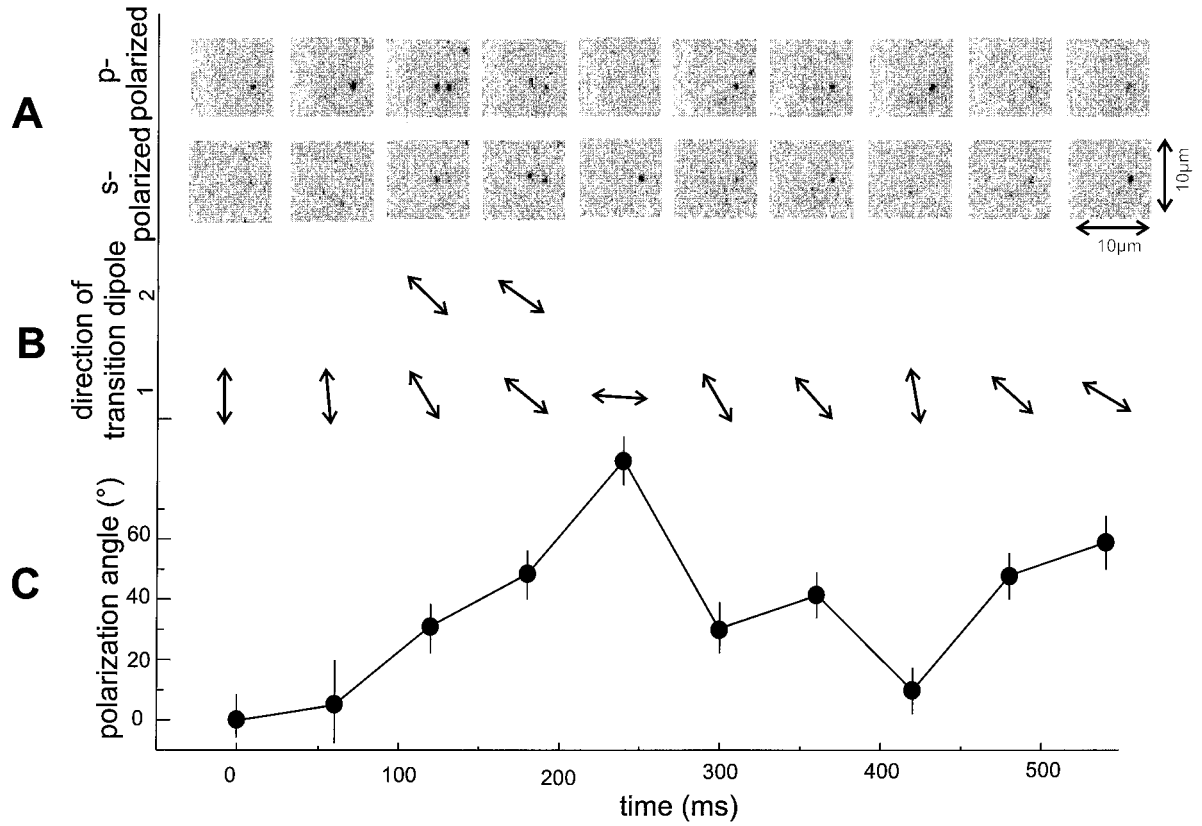


FIGURE 4 (*A*) Consecutively exposed p- and s-polarized image sequence of a $10 \times 10\ \mu m^2$ membrane area showing two individual TMR:POPE molecules in a DPPC lipid membrane at 20°C. The exposure time of the images was 10 ms, with a 50-ms time delay between exposures. The images were scaled from white (0 counts) to black (60 counts). One of the two molecules showed a "blinking" behavior often found in single-molecule studies (Dickson et al., 1997) (not further discussed in this paper). (*B*) Direction of the transition dipole moment of the molecules shown in *A*. The angle was determined from $\tan^2\phi = I_p/I_s$, where $I_p$ and $I_s$ denote the intensities in the p- and s-channels, respectively. (*C*) Rotational trajectory of one of the molecules shown in *A*.

ence in the direction of the transition dipole moments in absorption and emission, respectively, was neglected because of the high measured anisotropy here in DPPC membranes and that found in other references reporting the anisotropy of the fluorophores in viscous media (Timbs and Thompson, 1990; Schaffer et al., 1999). In such a way the functional relationship of $D_{rot}\tau$ on the two-dimensional anisotropy was determined.

Using the results of the Monte Carlo simulations, the data shown in Fig. 3, *A* and *B*, yielded $D_{rot}\tau = 0.16 \pm 0.01$ rad$^2$ for TMR-labeled and $D_{rot}\tau = 0.03 \pm 0.02$ rad$^2$ for Cy7-labeled lipids. Assuming fluorescence lifetimes of $\tau = 2.1$ ns for TMR (Soper et al., 1993) and 0.8 ns for Cy7 (M. Sauer, private communication), the rotational diffusion constants were $D_{rot} = 7 \pm 1 \times 10^7$ rad$^2$/s for TMR and $D_{rot} = 4 \pm 3 \times 10^7$ rad$^2$/s for Cy7. The value for Cy7-labeled lipids should be taken lightly, because the excited-state lifetime is much shorter than the inverse rotational diffusion constant. Anisotropy values greater than 0.4 are very sensitive to system noise, and slight imperfections of the polarization optics explain the large error for $D_{rot}$ found for Cy7. However, the values of $D_{rot}$ are in good agreement with values determined for lipid rotation by spin resonance techniques (Selig, 1977; Shin and Freed, 1989), which shows that the rotation of the fluorophores closely reflects the rotation of the phospholipids. In comparison to optical measurements on large ensembles (Timbs and Thompson, 1990, 1993; Bernsdorff et al., 1997), it should be stressed that, in addition to the mean anisotropies reported by single-molecule microscopy employed here, the distribution of anisotropies was determined.

An important result of the experiments presented is that rotational and lateral mobilities were determined simultaneously on the same molecule. Following the description by Saffman and Delbrück (1975) of membrane mobility, the two quantities are expected to be coupled by the membrane viscosity $\eta_m$. Thus knowledge of $D_{rot}$ allows an estimation of the lateral diffusion constant, $D^r_{lat,}$ on the time scale of the anisotropy measurement being characterized by the excited-state lifetime of the fluorophore. According to Saffman and Delbrück (1975),

$$D^r_{lat} = D_{rot}a^2\left[\ln\frac{\eta_m}{\eta_w a} - \gamma\right],$$

where the viscosity of the buffer, $\eta_w = 1$ cPoise; size of the lipid headgroup, $a = 3.8$ Å; Euler constant $\gamma$; and viscosity of a free-standing phospholipid membrane, $\eta_m = 7 \pm 1 \times$

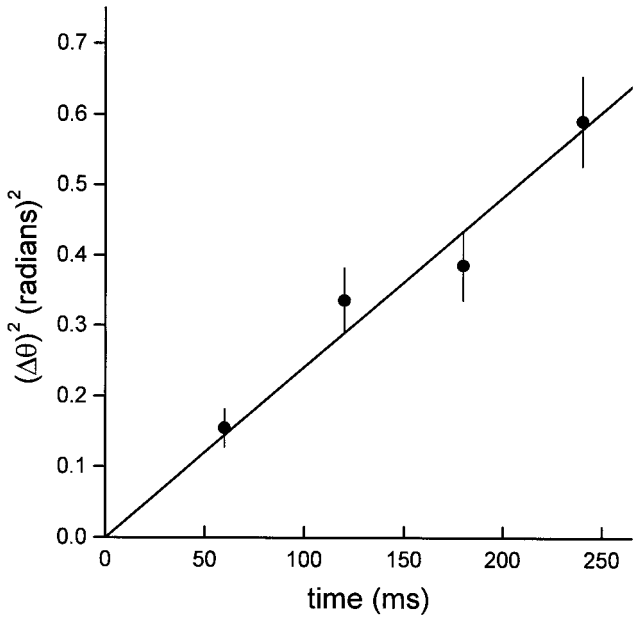


FIGURE 5 Ensemble average of the mean square angular displacement, $\langle\Delta\phi^2\rangle$, as a function of time, obtained from rotational trajectories of individual molecules of TMR:POPE in DPPC. The solid line represents a fit according to $\langle\Delta\phi^2\rangle = 2D_{rot}t$, yielding $D_{rot} = 1.2 \pm 0.1$ rad$^2$/s.

$10^{-8}$ Poise cm (Sonnleitner et al., manuscript submitted for publication). For the value of $D_{rot}$ measured, a lateral diffusion constant of $D^r_{lat} = 1.7 \times 10^{-6}$ cm$^2$/s is calculated. The value determined directly from the single-molecule trajectories is two orders of magnitude smaller, $D_{lat} = 3.5 \times 10^{-8}$ cm$^2$/s. The obvious discrepancy is due to the six-order-of-magnitude difference in time scale and the related three-order-of-magnitude difference in length scale of the two results (Shin et al., 1991; Vaz and Almeida, 1991). Whereas $D^r_{lat}$ describes the high mobility of a lipid molecule by probing its free volume on nanosecond time scales, the macroscopic mobility described by $D_{lat}$ is limited by the time of free volume formation on the order of tens to hundreds of nanoseconds (Vaz and Almeida, 1991).

For the fluid membrane the rotational motion was found to be on the same time scale as the fluorescence lifetime. The latter statement is different for phospholipid membranes in the $L_\beta$ gel state. It is known that the lipid DPPC forms gel-like membranes at room temperature characterized by a lateral diffusion constant of $D_{lat} < 10^{-11}$ cm$^2$/s (Timbs and Thompson, 1990, 1993). The two-dimensional fluorescence anisotropy was $\langle r_{lin}\rangle = 0.41 \pm 0.05$ for TMR-labeled lipids as determined from 440 individual molecules, and $\langle r_{lin}\rangle = 0.39 \pm 0.10$ for Cy7-labeled lipids obtained from 137 molecules (Fig. 3, *C* and *D*). The high values of $r$ for both fluorophores show that the rotational diffusion is much slower than the fluorescence lifetime. Values for the rotational diffusion constants for such high values in $r$ can only be estimated with $D_{rot} < 10^7$ rad$^2$/s, because the inherent uncertainty in the determination of the fluorescence intensity from a single molecule (Schmidt et al., 1996) prohibits a more precise determination of $D_{rot}$ by the method outlined for the fluid membrane.

However, if the rotational diffusion is on the order of the observation time (ms here), the rotation of the molecules can be visualized directly (Ha et al., 1996, 1998; Schütz et al., 1997a). It should be noted that this ability is a unique property of experiments at the level of individual molecules. Fig. 4 shows the development of the fluorescence polarization of an individual TMR-labeled phospholipid molecule on a DPPC membrane. The membrane was illuminated for 10 ms per image by circularly polarized light, and images were taken with a delay of 50 ms. The slow rotation of the fluorescence polarization interpreted by a slow rotation of the transition dipole moment is clearly seen. For quantitative interpretation the direction of the transition dipole moment in each pair of images was calculated from the relation $\tan^2\phi = I_p/I_s$ (see indicators in Fig. 4), where $I_p$ and $I_s$ are the single-molecule fluorescence intensities corrected by $g$. The angular trajectory is displayed at the bottom of Fig. 4. (In the determination of the direction of the transition dipole moment, $\phi$, it was assumed that the angle always falls into the first quadrant of the polar coordinate system. The unavoidable multiplicity of equivalent angular values $n\pi \pm \phi$ ($n$ integer) was further neglected. This multiplicity could be overcome by simultaneous rotation of the excitation polarization (Ha et al., 1996, 1998; Schütz et al., 1997a).) It should be noted that even a single observation of an individual molecule is sufficient to determine its orientation in the plane with an uncertainty of ~5° (given by the uncertainties in the intensity determination).

Angular trajectories such as those shown in Fig. 4 were further evaluated by using algorithms developed for lateral diffusion. For all combinations of delay times the squared angular displacements were determined. These data yielded the time dependence of mean squared angular displacements, $\langle\Delta\phi^2\rangle$, in Fig. 5. $\langle\Delta\phi^2\rangle$ increases linearly with time according to the relation for one-dimensional rotational diffusion (Chandrasekhar, 1943), $\langle\Delta\phi^2\rangle = 2D_{rot}t$. The corresponding fit, shown as a solid line, is characterized by the rotational diffusion constant of $D_{rot} = 1.2 \pm 0.1$ rad$^2$/s for the TMR-labeled and $D_{rot} = 2.0 \pm 0.2$ rad$^2$/s for the Cy7-labeled (data not shown) lipid molecules in DPPC.

In conclusion, studies on the level of individual molecules have made possible the simultaneous observation of lateral and rotational mobilities of a membrane component. There are two areas of most significance for this technique. First, rotational mobility on the time scale of the excited-state lifetime (~ns) can be determined by a time-averaged approach. Because the rotational mobility of a molecule is highly sensitive to the phase state of the surrounding membrane, steady-state anisotropy measurements allow for the determination of local heterogeneities or phase separation commonly found in biological systems. Second, rotation on the millisecond time scale can be visualized directly. The latter is expected to have the potential of direct observation of conformational motions of peptides, DNA, and proteins,

which are indicative of structural changes necessary to biological reactions.

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