Probing Diffusion Laws within Cellular Membranes by Z-Scan Fluorescence Correlation Spectroscopy

Jana Humpolíčková,* Ellen Gielen,†‡ Aleš Benda,* Veronika Fagulova,* Jo Vercammen,† Martin vandeVen,‡ Martin Hof,* Marcel Ameloot,‡ and Yves Engelborghs†

*J. Heyrovský Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic; †University of Leuven, Biomolecular Dynamics, Leuven, Belgium; and ‡Biomedical Research Institute, Hasselt University and Transnationale Universiteit Limburg, Diepenbeek, Belgium

ABSTRACT The plasma membrane of various mammalian cell types is heterogeneous in structure and may contain microdomains, which can impose constraints on the lateral diffusion of its constituents. Fluorescence correlation spectroscopy (FCS) can be used to investigate the dynamic properties of the plasma membrane of living cells. Very recently, Wawrezinieck et al. (Wawrezinieck, L., H. Rigneault, D. Marguet, and P. F. Lenne. 2005. *Biophys. J.* 89:4029-4042) described a method to probe the nature of the lateral microheterogeneities of the membrane by varying the beam size in the FCS instrument. The dependence of the width of the autocorrelation function at half-maximum, i.e., the diffusion time, on the transverse area of the confocal volume gives information on the nature of the imposed confinement. We describe an alternative approach that yields essentially the same information, and can readily be applied on commercial FCS instruments by measuring the diffusion time and the particle number at various relative positions of the cell membrane with respect to the waist of the laser beam, i.e., by performing a Z-scan.

Received for publication 19 May 2006 and in final form 26 May 2006.

J. Humpolíčková and E. Gielen contributed equally to this work.

Address reprint requests and inquiries to Yves Engelborghs, Tel.: 32-16-32-7160; E-mail: yves.engelborghs@fys.kuleuven.be.

Due to a variable degree of lipid miscibility (1), cellular membranes contain a variety of molecular complexes and domains, characterized by different composition and spatial arrangement of the lipids constituting the membrane. One class of membrane inhomogeneities are the so-called lipid rafts. These liquid-ordered microdomains, built mainly of cholesterol and saturated lipids, attract a lot of interest as they are thought to provide dynamic platforms that are involved in a variety of processes, such as signal transduction and protein and lipid sorting (2).

Most of the experimental evidence concerning the existence of rafts in cellular membranes comes from biochemical studies, which show that some membrane constituents are resistant to solubilization by nonionic detergents at low temperature (3). However, as application of a detergent can alter the membrane phase behavior (4), alternative in situ methods are required.

Another type of membrane inhomogeneity is the confined diffusion of membrane constituents within membrane compartments made up by the membrane-associated actin cytoskeleton (fences) and by rows of transmembrane proteins anchored to it (pickets) (1).

Several microfluorimetric methods (5) have been applied to study the dynamics of membrane constituents within the context of the lateral microheterogeneity of the cellular membrane. In fluorescence correlation spectroscopy (FCS) small fluctuations in the fluorescence signal from a femtoliter observation volume are measured over a short period of time. These fluctuations arise from fluorescently labeled molecules diffusing in and out of the observation volume, which is spatially defined by the laser focus. The corresponding autocorrelation function (ACF) contains information about the average number of molecules in the observation volume and their characteristic diffusion time as measured by the full width at half-maximum of the ACF (6). It was recently demonstrated that FCS has the potential to be a valuable tool in the study of lipid raft associations, both in model membranes exhibiting domains and in cell membranes (7,8).

For hindered or confined diffusion and for the case where the area of confinement is several times smaller than the beam area, the relation between the diffusion time τ_D and the beam area is given by (8)

$$\tau_D = t_0 + w^2(4D_{eff})^{-1}, \quad (1)$$

where w is the radius of the beam at the plane of diffusion, D_{eff} the so-called effective diffusion coefficient, and t_0 a constant. For free diffusion, t_0 is equal to zero. A nonzero value for t_0 is indicative of hindered diffusion and is proportional to the confinement time within the microdomains. If $t_0 > 0$, the confinement is due to rafts; if $t_0 < 0$, the confinement is due to a meshwork (8).

doi: 10.1529/biophysj.106.089474

The consequence of these observations is that single-waist FCS measurements are not sufficient in the case of hindered diffusion and measurements at different waist sizes are necessary. This requires either a beam expander or underfilling of the objective, which may be a technical problem for most commercial FCS instruments. In this letter, we introduce an alternative based on the Z-scan method. This approach was used for precise determination of the diffusion coefficient in supported phospholipid bilayers (SPBs) (9). The method is based on acquiring consecutive sets of FCS measurements along the z axis of the instrument.

For a Gaussian beam, the radius w can be expressed as (10)

$$w^2 = w_0^2\left(1 + \frac{\lambda_0^2\Delta z^2}{\pi^2 n^2 w_0^4}\right), \tag{2}$$

where w_0 is the radius of the beam in the waist, λ_0 is the wavelength of the excitation light in vacuum, n is the refractive index of the medium, and Δz is the distance between the sample position and the position of the minimum laser-beam diameter. The average number of particles N in the illuminated membrane surface within the confocal volume at each value of Δz is given by

$$N = N_0\left(1 + \frac{\lambda_0^2\Delta z^2}{\pi^2 n^2 w_0^4}\right), \tag{3}$$

where $N_0 = \pi c w_0^2$ and c is the average concentration of diffusing fluorescent molecules in a plane perpendicular to the beam. For free diffusion with diffusion coefficient D, the z-dependence of the diffusion time is given by

$$\tau_D = \frac{w_0^2}{4D}\left(1 + \frac{\lambda_0^2\Delta z^2}{\pi^2 n^2 w_0^4}\right). \tag{4}$$

From the known dependencies of τ_D and N on the z-position of the plane, the lateral diffusion coefficient and the surface concentration can be obtained without the need for an extrinsic calibration. The method generally allows for precise positioning of the membrane in the waist of the beam and increases the accuracy of the measurements.

For the case of confined diffusion, the relation between τ_D and N is given by

$$\tau_D = t_0 + \frac{w_0^2}{4D_{\text{eff}}}\frac{N}{N_0}. \tag{5}$$

Equation 5 is formally equivalent to Eq. 1 and implies a parabolic dependence of the diffusion time on Δz.

We explored the Z-scan method to evaluate the diffusion of the membrane marker 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) (Molecular Probes, Eugene, OR) in SPBs (11) and in the plasma membrane of OLN-93 cells (oligodendroglioma derived from primary rat brain glial culture (12)).

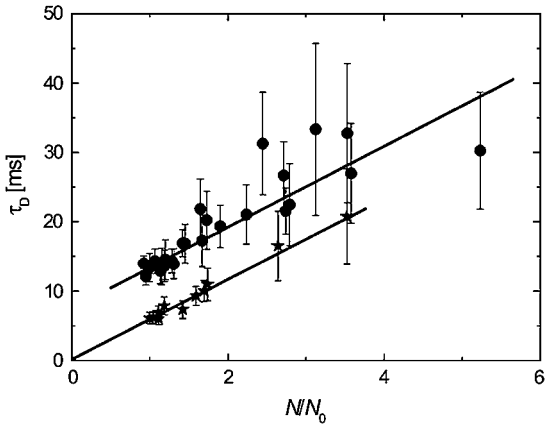

FIGURE 1 Results obtained for DiD in DOPC:DOPS SPBs on mica (★) and in OLN-93 (●). Error bars represent standard errors.

SPBs were prepared from small unilamellar vesicles (SUVs) resulting from sonication of multilamellar vesicles obtained from resuspending a dried mixture of DOPC and DOPS (DOPC/DOPS = 4:1). Labeled SUVs were prepared in the presence of DiD (lipid/dye = 10,000:1). Mica slides were fixed to a microscrew and positioned in a cuvette. Labeled and nonlabeled SUVs were mixed (1:9), and 50 μL of the mixture was added to the cuvette. After at least 30 min, the SPB was formed and remaining SUVs were washed away.

OLN-93 cells were grown in eight-well glass LabTek chambers (Nalge Nunc, Rochester, NY) in growth medium (GM). Cells were labeled with DiD (5 μM DiD in GM for 15 min at 37°C). FCS measurements were performed in HEPES buffered phenol red-free medium supplemented with 10% serum. The measurements were performed at the membrane facing the supporting glass. All data were corrected for the constant background of 1 kHz.

FCS and confocal imaging were carried out on a Zeiss Confocor 2 microscope (Carl Zeiss Microscopy, Jena, Germany) with a 40×, NA 1.2 water immersion objective. The pinhole was set at 90 μm. Samples were excited with a 633 nm He-Ne laser at 0.1% of the maximum laser power to prevent photobleaching as much as possible. In addition to the triplet formation of DiD the *cis-trans* transition was also taken into account. The ACF $G(\tau)$ is given by

TABLE 1 Effective diffusion coefficients of DiD in SPBs and OLN-93 cells obtained from a Z-scan at room temperature

	t_0 (ms)	D_{eff} (μm^2 s^{-1})
SPB	0.2 ± 1.6	5 ± 1
SPB	0 (fixed)	4.4 ± 0.3
OLN-93	8 ± 1	4.5 ± 0.8

Obtained from weighted linear least-squares fitting using Eq 5; uncertainties are reported as standard error.

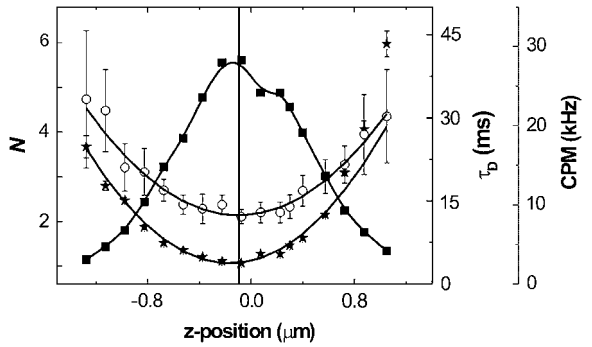

FIGURE 2 Results obtained for DiD in OLN-93. CPM is counts per second per molecule. CPM, ■; τ_D, ○; and *N*, ★. Error bars represent standard errors.

$$G(\tau) = 1+\frac{1}{N}\frac{1}{1+\frac{\tau}{\tau_D}}\left[1+\sum_{i=1}^{2}\frac{T_i}{1-T_1-T_2}\exp\left(-\frac{\tau}{\tau_{0i}}\right)\right], \quad (6)$$

where τ_{01} and τ_{02} are the inverse of the triplet rate constant and the *cis-trans* transition rate constant, respectively; and T_1 and T_2 are the corresponding amplitude fractions.

The results obtained on SPBs (Fig. 1 and Table 1) clearly indicate that DiD in the SPBs exhibits free diffusion.

The waist of the laser was estimated by using the parabolic fit ($w_0 = 0.323 \pm 0.008$ μm) and by applying an extrinsic calibration using Cy5 (Amersham Biosciences, Uppsala, Sweden) in water and assuming a diffusion coefficient of 316 $\mu m^2/s$ ($w_0 = 0.335 \pm 0.010$ μm). The values of the diffusion coefficient of DiD correspond with those reported in the literature (13).

The results obtained for OLN-93 are shown in Figs. 1 and 2 and in Table 1. It has to be emphasized that good fits were obtained by using Eq. 6 for each of the ACF collected at the various values of Δz. The value for t_0 is significantly greater than zero, suggesting hindered diffusion by rafts, according to the model suggested by Wawrezinieck et al. (8). Similar results were obtained in 15 independent cell measurements.

In conclusion, it can be stated that single-waist FCS measurements do not allow for a complete description of the lateral diffusion in the cellular membrane. Plotting the apparent diffusion times versus the relative particle number, which is a measure of the illuminated area, clearly indicates hindered diffusion. The proposed Z-scan yields the same type of information as that obtainable by changing the waist of the laser beam (8) and can be applied on commercial FCS instruments. The additional benefits are that the Z-scan approach implicitly considers optimal position of the membrane in the focus volume, leads to improved precision of the recovered parameters, and unreliable measurements are easily identified.

ACKNOWLEDGMENTS

We thank Prof. Dr. C. Richter-Landsberg, Molecular Neurobiology Department, Oldenburg University, Germany, for the OLN-93 oligodendroglial cells.

This work has been supported by the Research Council of the UHasselt and tUL, the K.U. Leuven (grant No. GOA/2006/02), a bilateral project (grant No. BIL03/08) between Flanders and the Czech Republic, the Grant Agency of the Czech Republic (A.B. and M.H. via grant No. 203/05/2308), and the Ministry of Education, Youth and Sports of the Czech Republic (J.H. and V.F. via grant No. LC06063).

REFERENCES and FOOTNOTES

1. Kusumi, A., I. Koyama-Honda, and K. Suzuki. 2004. Molecular dynamics and interactions for creation of stimulation-induced stabilized rafts from small unstable steady-state rafts. *Traffic*. 5:213–230.
2. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature*. 387:569–572.
3. Brown, D. A., and J. K. Rose. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell*. 68:533–544.
4. Heerklotz, H. 2002. Triton promotes domain formation in lipid raft mixtures. *Biophys. J*. 83:2693–2701.
5. Lommerse, P. H., H. P. Spaink, and T. Schmidt. 2004. In vivo plasma membrane organization: results of biophysical approaches. *Biochim. Biophys. Acta*. 1664:119–131.
6. Haustein, E., and P. Schwille. 2004. Single-molecule spectroscopic methods. *Curr. Opin. Struct. Biol*. 14:531–540.
7. Bacia, K., D. Scherfeld, N. Kahya, and P. Schwille. 2004. Fluorescence correlation spectroscopy relates rafts in model and native membranes. *Biophys. J*. 87:1034–1043.
8. Wawrezinieck, L., H. Rigneault, D. Marguet, and P. F. Lenne. 2005. Fluorescence correlation spectroscopy diffusion laws to probe the submicron cell membrane organization. *Biophys. J*. 89:4029–4042.
9. Bendá, A., M. Benes, V. Marecek, A. Lhotsky, W. T. Hermens, and M. Hof. 2003. How to determine diffusion coefficients in planar phospholipid systems by confocal fluorescence correlation spectroscopy? *Langmuir*. 19:4120–4126.
10. Sorscher, M. S., and M. P. Klein. 1980. Profile of a focused collimated laser beam near the focal minimum characterized by fluorescence correlation spectroscopy. *Rev. Sci. Instrum.* 51:98–102.
11. Spink, C. H., M. D. Yeager, and G. W. Feigenson. 1990. Partitioning behavior of indocarbocyanine probes between coexisting gel and fluid phases in model membranes. *Biochim. Biophys. Acta*. 1023:25–33.
12. Richter-Landsberg, C., and M. Heinrich. 1996. OLN-93: a new permanent oligodendroglia cell line derived from primary rat brain glial cultures. *J. Neurosci. Res*. 45:161–173.
13. Kahya, N., D. Scherfeld, K. Bacia, B. Poolman, and P. Schwille. 2003. Probing lipid mobility of raft-exhibiting model membranes by fluorescence correlation spectroscopy. *J. Biol. Chem*. 278:28109–28115.