Biochimica et Biophysica Acta 1788 (2009) 225–233

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Fluorescence correlation spectroscopy in membrane structure elucidation

Salvatore Chiantia, Jonas Ries, Petra Schwille *

Biotechnologisches Zentrum (BIOTEC), TU Dresden, Germany

ARTICLE INFO

Article history:
Received 2 July 2008
Received in revised form 12 August 2008
Accepted 21 August 2008
Available online 29 August 2008

Keywords:
Membrane dynamics
FCS
Anomalous diffusion
Raft
Picket model
Fence model

ABSTRACT

This review describes the application of fluorescence correlation spectroscopy (FCS) for the study of biological membranes. Monitoring the fluorescence signal fluctuations, it is possible to obtain diffusion constants and concentrations for several membrane components. Focusing the attention on lipid bilayers, we explain the technical difficulties and the new FCS-based methodologies introduced to overcome them. Finally, we report several examples of studies which apply FCS on both model and biological membranes to obtain interesting insight in the topic of lateral membrane organization.

Contents

1. Introduction

Fluorescence correlation spectroscopy (FCS) is a technique based on the statistical analysis of the signal fluctuations detected from fluorophores in a very small volume (~fL). Introduced in 1973 by Magde et al. [1], this technique experienced a huge growth in popularity during the last years, especially since the demonstration of its single-molecule detection capability [2]. Nowadays, FCS is a commonly used tool to efficiently measure local concentrations, translational and rotational diffusion coefficients, photodynamics, reaction constants and molecular aggregation [3–6]. In order to satisfy the technical challenges met in the study of complex biological systems, variations and improvements of standard FCS are continuously being developed [7]. The aim of this review is to illustrate how FCS can be employed to investigate the properties of lipid bilayers, with a particular focus on the lateral structure of the plasma membrane and its physical models.

Cellular membranes are complex biological entities, far from being an inert assembly of proteins and lipids which just separates cells from the surrounding environment. A multitude of biological processes, ranging from controlled transfer of ions to immune response, are regulated at the level of the plasma membrane [8]. In order to perform these functions, a very large variety of lipids and proteins in cell membranes organize into a double-layer structure that was initially schematized as a two-dimensional fluid [9]. In contrast with the hypothesis of simple homogenous lipid mixing, recent evidences have shown that membranes are heterogeneously arranged both in the plane of the bilayer and across the two leaflets. For example, the lipid

* Corresponding author. Tatzberg 47-51, 01307 Dresden, Germany. Tel.: +49 351 46340328; fax: +49 351 46340342.
E-mail addresses: chiantia@biotec.tu-dresden.de (S. Chiantia), petra.schwille@biotec.tu-dresden.de (P. Schwille).

0005-2736/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2008.08.013

bilayer of eukaryotic cell membranes is asymmetric, the outer leaflet being enriched in sphingolipids like phosphatidylcholine (PC) and the inner leaflet enriched in phosphatidylserine (PS) or phosphatidylethanolamine (PE) [10]. In epithelial cells, the plasma membrane is distinguished in basolateral and apical domains, the former being enriched in PC and the latter in sphingolipids [11]. Furthermore, it was shown that caveolae – small invaginations in the plasma membrane – are also characterized by high concentration of glycosphingolipids [12]. These and several other findings [13–15] have led to the more general hypothesis that cell membranes contain small microdomains called rafts, enriched in cholesterol, sphingolipids and a certain subset of membrane proteins[16]. If the rest of the plasma membrane can be assumed to be in a liquid-disordered (Ld) state at physiological temperatures, these hypothetical lipid domains might consist of lipids in a liquid-ordered (Lo) state [17]. While the steady-state existence, size and shape of ordered domains in living cells is still subject of debate [18,19], agreement has been reached on the fact that native cell membranes could exhibit phase separated domains [20–23]. Such domains appear and/or coalesce upon stimulation, forming signaling and sorting platforms [24–26].

Lipid–protein microdomains are not the only examples of lateral inhomogeneities that might be present in the plasma membrane. Based on the specific dynamics observed for single proteins and lipids, it was proposed that the cytoskeleton underlying the membrane can directly or indirectly confine the motion of membrane components (see the clarifying review by Kusumi et al. [27] and Section 5).

An exhaustive discussion about the existence of lipid domains in the plasma membrane and their relation to the model of cytoskeleton-dependent confinement is beyond the scope of this review. Nevertheless, it appears clear that the inhomogeneities in lateral structure of the lipid bilayer can be related to a non random distribution and lateral segregation of specific lipids and receptors. Importantly, changes in the local concentration and dynamics of membrane components are essentially connected to the multitude of biological processes associated to the cellular membrane [17,25,28–38]. In this context, FCS can be used to investigate the local concentration, the segregation or oligomerization state and, more generally, the diffusion properties of membrane components, therefore providing an important tool for the study of membrane structure and biological activity. Compared to fluorescence recovery after photobleaching (FRAP) [39,40], a very commonly used technique to measure diffusion in membranes, FCS requires a concentration of fluorescent probes and laser powers orders of magnitude lower [41]. Since membrane heterogeneities might be fragile structures prone to preparation artifacts, such an optical method causing negligible perturbation of the sample provides a promising approach [42]. Compared to very sensitive single-molecule techniques, like single-molecule tracking (SMT) or single particle tracking (SPT) [43–45], FCS analysis offers reliable statistics and immediate experimental readout, without time consuming offline data analysis. As a drawback, information about single-molecule behavior (e.g. specific trajectories, temporary confinement) [46,47] might be lost. A quantitative comparison between FCS and other methods probing diffusion behavior in membranes can be found in the work by Guo et al. [48].

In this article, we first describe the basic principles of FCS and its practical application for the study of biological membranes. After commenting on possible experimental problems and artifacts, we present and discuss recent work involving the use of this technique in the context of the lipid domains in model membranes and complex dynamics observed for membrane components in living cells.

2. Theory of fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) relies on the statistical analysis of fluorescence intensity fluctuations in a system in thermodynamic equilibrium. In confocal FCS, a laser is focused by the microscope objective to a diffraction limited spot. A pinhole in the emission channel provides tight axial confinement resulting in a small (~fL) detection volume (Fig. 1A). Diffusion of fluorophores through the detection volume and photophysical or photochemical reactions causes fluctuations in the detected emission (Fig. 1B). The intensity trace $F(t)$ is analyzed by calculating the auto-correlation curve (Fig. 1C), which measures the self-similarity of the signal as a function of time:

$$G(\tau) = \frac{\langle \delta F(t)\delta F(t+\tau)\rangle}{\langle F(t)\rangle^2} \tag{1}$$

Here $\langle\ \rangle$ denotes the time average,

$$\delta F(t) = F(t) - \langle F(t)\rangle. \tag{2}$$

τ is called the lag time.

The experimental auto-correlation curve is then fitted with a mathematical model function to extract the parameters of interest, such as the diffusion coefficient D or the concentration C. For measurements in solution, the detection volume is usually approximated by a three-dimensional Gaussian profile. The correlation function describing three-dimensional Brownian diffusion through such a profile is:

$$G(\tau) = \frac{1}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\left(1+\frac{\tau}{S^2\tau_D}\right)^{\frac{1}{2}}. \tag{3}$$

$N = CV_{eff}$ is the number of particles in the detection volume $V_{\text{eff}} = \pi^{\frac{3}{2}} S w_{xy}^3$; the form factor $S = \frac{w_z}{w_{xy}}$ measures the aspect ratio of

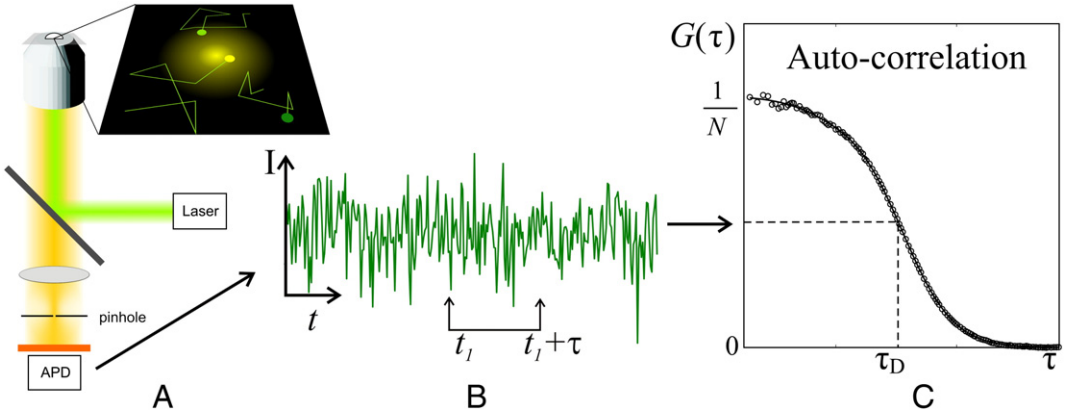

Fig. 1. Principle of FCS illustrated through the detection setup (A), the recording of fluorescence fluctuations (B) and the calculation of the auto-correlation of the signal (C). See text for details. Adapted from Ref. [7].

the elliptic detection volume. The diffusion time $\tau_D = \frac{w_{xy}^2}{4D}$ is the decay time of the correlation curve. To extract the concentration C or the diffusion coefficient D from the relative parameters N or τ_D, the size of the laser focus w_{xy} has to be known. This is usually determined by a calibration measurement of a dye with a known diffusion coefficient [3].

For measurements on membranes, the intersection of the laser with the membrane defines the two-dimensional Gaussian detection area. The corresponding correlation function is:

$$G(r) = \frac{1}{N}\left(1 + \frac{\tau}{\tau_D}\right)^{-1}. \quad (4)$$

$N = A_{\text{eff}}C$ is the number of particles in the detection area and C is the area concentration.

In general, photophysical phenomena (e.g. triplet transitions, blinking) and several emitting species with different diffusion properties might have to be taken into account. Systems with submicroscopic heterogeneities exhibit a non-Brownian, anomalous diffusion which is described by a different correlation curve (see Section 5). Specific interactions between individually labeled partners (e.g. two membrane proteins) can be studied with fluorescence cross-correlation spectroscopy (FCCS). With this FCS variation, the similarity between the fluorescence fluctuations in two separate detection channels is evaluated, thus monitoring the binding and collective motion of different membrane components [5]. FCCS is mainly used to detect protein–protein interaction both in model [49] and cell membranes [50,51], rather than investigating the structure of the membrane itself. For more detailed information, the reader is referred to Bacia et al. [5,42,52,53].

3. FCS on lipid membranes

Although FCS is an established technique to study diffusion in solution, several additional problems connected with slow dynamics and two-dimensional geometry strongly limit the accuracy and even applicability of confocal FCS on membranes. In this paragraph, we will give an overview about these problems and the novel technical developments to avoid them. For a detailed coverage of this topic, see also the previous review by Ries and Schwille [7].

In confocal FCS, the detection volume has to be positioned in the membrane with a vertical accuracy of approximately 100 nm. Otherwise, the divergence of the laser would lead to an unwanted enlargement of the effective detection area. Also, optical artifacts (e.g. saturation, cover slide thickness local variations, refractive index mismatch, astigmatism [54]) cause distortions of the focal volume, impeding the accurate determination of the detection area which is necessary for quantitative measurements. Therefore, FCS techniques which do not rely on the calibration of the detection area are highly recommended for quantitative and reliable measurements on membranes.

The diffusion in lipid membranes can be several orders of magnitude slower than that of fluorophores diffusing freely in solution. To average over a significant number of independent events, long measurement times, at least 10^4 times the diffusion time, are necessary [7]. During this time, the position of the detection volume with respect to the membrane has to be stable within about 100 nm; otherwise additional changes in the intensity trace are introduced with consequent distortion of the correlation curve. Even minor instabilities might result in an apparent second component or non-Brownian diffusion. To measure slow diffusion, one can either use techniques which allow the correction for instabilities [55] or techniques where parallel acquisition decreases the measurement times [56,57].

Another consequence of the slow diffusion observed in membranes is the long residence time of the fluorophores in the detection volume. This leads to strong photobleaching, resulting in an apparent reduction of the measured diffusion times. Therefore low laser powers have to be used for membrane FCS. Unfortunately, low excitation power produces a weak signal, easily concealed by stray light or detector dark counts, and thus requires long measurement times.

On membranes, the two-dimensional geometry precludes an efficient replenishment of bleached fluorophores which are depleted in the detection area. Initially, the intensity trace quickly decays with time, resulting in distorted correlation curves. Starting the measurement after the system has reached a quasi-steady state avoids distortions but leads to an underestimation of the concentration. Depletion is especially problematic if the reservoir of fluorophores is limited and a quasi-steady state cannot be reached. This is the case for small domains in phase separating membranes or for closed bilayer and cell membranes. Depletion due to photobleaching can be corrected at the level of the intensity trace [56].

Even moderate excitation laser powers can induce non-radiative energy dissipation phenomena, like electrochemical alteration of the lipids or heat production. This may cause a significant alteration of the physical properties of the membrane [58]. We often observed ordered-to-disordered transition in model membranes at the exact location of the laser focus. Fig 2A shows a fluorescence image of a Lo domain in a SM/DOPC/cholesterol model membrane (see Section 4 for more details on the specific system) surrounded by lipids in the Ld phase. The red channel represents the signal from the fluorescent lipid analogue DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) in the Ld phase, and the green channel that of the fluorescent B subunit of Cholera Toxin bound to the monosialotetrahexosylganglioside (GM1) in the Lo

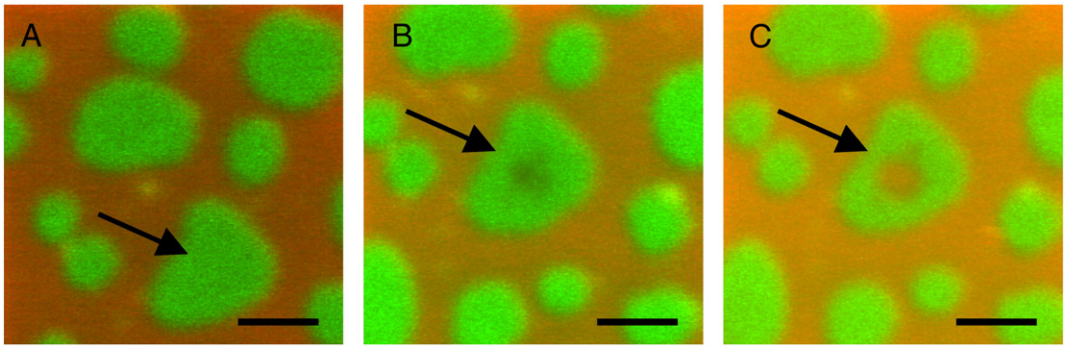

Fig. 2. Light-induced Lo to Ld transition. (A) Laser scanning fluorescence imaging of a supported lipid bilayer composed of SM, DOPC 1:1 molar and 25% molar cholesterol. Alexa-488 labeled Cholera toxin, 0.1% molar GM1 and DiD were added for fluorescence imaging and FCS measurements. The Ld lipid phase is visualized in red (DiD) and the Lo phase in green (Alexa-488 Cholera toxin bound to GM1). The arrow indicates the exact localization of the focus during FCS measurements. The employed laser power was 2–4 μW (488 nm). (B) After FCS measurements (see text), a bleached spot appears in the Lo domain (i.e. dark in both the red and green channels). (C) After few minutes, replenishment of the bleached spot can be observed. The spot can be visualized in the red channel only, indicating the presence of Ld phase. Scale bar = 2 μm.

phase. The concentration of GM1 was 0.1% molar. After a 10 second FCS measurement with moderate laser power in the Lo domain pointed by the arrow, we observed strong bleaching of the Cholera toxin (Fig. 2B) and, after few minutes, local enrichment of the Ld-marker DiD (Fig. 2C). These images, together with the 2-component dynamics observed in the FCS measurement (data not shown), demonstrates the possibility of localized light-induced melting of the ordered phase. If a reduction of the fluorophore concentration and excitation power does not avoid this artifact, FCS with a stationary detection volume should not be used.

In many experiments, a fluorescent background in the solution around the membrane cannot be avoided. Measurements in cellular membranes are precluded by intracellular fluorescence and, especially, by fluorescent vesicles in the cytosol which have a similar diffusion time compared to molecules diffusing in the more viscous membrane. When studying a molecule with a low binding affinity to the membrane, the high concentration in solution required for significant binding can completely mask the signal originating in the membrane. To reduce the fluorescent background, very flat detection volumes which are provided by FCS with total internal reflection excitation (TIRF-FCS) [59] or with FCS using a supercritical angle objective [60] have to be employed.

3 1. Calibration-free FCS

Since it is difficult to exactly infer the size of the detection area, calibration-free techniques, which do not rely on the knowledge of this parameter, greatly increase the accuracy of quantitative measurements on membranes. The idea is to introduce an 'external ruler', an additional spatial parameter which can be very accurately determined. For the fitting of the experimental data, the size of the detection area can then be treated as a free parameter. Calibration-free techniques useful to study membrane dynamics are e.g. z-scan FCS [61,62], two-focus FCS [63,64] and scanning FCS [55,56,65,66].

The z-scan method is easy to implement, since it does not require any additional hardware. In this case, correlation curves must be acquired at well defined axial positions. The divergence of the laser leads to increased diffusion times and particle numbers for larger distances from the membrane. For a focused laser beam, there is a unique correspondence between the divergence of the beam and the size of the focus w_{xy}. Therefore, an analysis of the axial dependence of the diffusion times and particle numbers allows the determination of the diffusion coefficients and concentrations on the membrane without an additional calibration measurement. For slow diffusion, the z-scan method is only limited by instabilities of the setup, since not only one, but many correlation curves at well known axial positions have to be obtained.

In two-focus FCS, two detection volumes are employed, their distance serving as the external ruler. Diffusion coefficients, concentrations and the size of the detection area result directly from the fit. The use of alternating excitation is mandatory to avoid spatial cross-talk. Two foci can be produced from orthogonal lasers with a DIC-prism [64,67] or by scanning two parallel lines [55,63]. The detection of two foci with a CCD camera allows the determination of their distance directly with a high accuracy [68].

In spatiotemporal image correlation spectroscopy [57], the spatial correlation curves contain the information about the size of the detection volume. In addition, the parallel acquisition of a whole frame greatly increases the statistical accuracy. However, the still rather long time required to acquire a full frame limits the temporal resolution and the accuracy of diffusion measurements. By scanning only a line instead of a whole frame [56], also fast membrane diffusion can be accurately measured. The short residence time of the fluorophores in the scanned detection volume leads to greatly reduced photobleaching and allows the use of high excitation laser powers to obtain a high signal to noise ratio. The implementation of scanning FCS with a scanning path perpendicular to the membrane plane [55] does not require a planar system. Therefore it can be used in spherical model membranes or cells and even in the membranes of multi-cellular organisms (J. Ries, S.R. Yu, P. Schwille, M. Brand, unpublished data). Instabilities can be corrected for, allowing for long acquisition times necessary to measure slow diffusion. Scanning FCS can be easily extended to two-focus scanning FCS for calibration-free diffusion measurements and to dual color scanning FCS with alternating excitation for cross-talk free determination of binding in the membrane.

3 2. Fluorophores for FCS in membranes

The careful choice of suitable fluorophores is essential for the success of a FCS experiment. There are, in fact, specific requirements that these molecule must meet: considerable partition into the lipid bilayer, high quantum efficiency, large absorption cross section, and photostability [69]. The last characteristic is particularly important for measurements in membranes because the fluorophores spend a long time in the focal volume (1–100 ms) if compared to the case of 3d diffusion measurements in solution (10–100 μs). Also, since photophysical phenomena like blinking and triplet state transitions are usually on the μs scale, they hardly influence the diffusion-related decay of the correlation curve and do not strongly matter in the choice of a good dye.

Among the commonly available alternatives for fluorescent membrane components, we report the family of long-chain dialkylcarbocyanines, like DiD, DiI, DiA and DiO (Invitrogen, Oregon). These are fluorescent lipid analogues, with a large range of excitation and emission wavelengths. They are characterized by well defined transition dipole moment parallel to the plane of the membrane and lipid moieties consisting of acyl chain with different lengths and degrees of saturation. Another possibility is to use lipids chemically labeled with fluorescent molecules like Rhodamine and Bodipy (Invitrogen and Avanti Polar Lipids, Alabaster) [70]. These lipids can be labeled on the hydrophilic head or, more commonly, on the hydrophobic chains. The former alternative might cause impairment of specific lipid activity (like in the case of the GM1-Cholera toxin binding [71]), while the latter might significantly alter its partition properties between different bilayer phases [63]. In this regard, it seems that the hydrophobicity of the fluorophore and the specific chemical characteristic of its linkage to the lipid may be important in determining the partition properties [72,73]. Also, the electrical charge of the specific fluorescent lipid might play a role in the choice of probes for FCS studies. In the case of supported membranes (both model and adherent cellular membranes), different fluorescent lipids in the same bilayer show different local mobilities due to electrostatic interaction with the support [63]. Finally, FCS can be also performed in membranes employing fluorescent proteins. It is a common approach to chemically label proteins using reactive dyes specific for cysteines or amine groups [74]. Examples of dyes suitable for protein labeling are Alexa, Rhodamine NHS (Invitrogen) or Atto (Atto-TEC, Siegen, Germany). In the case of in vivo measurements, it might be advantageous to genetically modify membrane proteins through the fusion to naturally fluorescent proteins like green fluorescent protein (GFP), red fluorescent protein (RFP), or mCherry. For an exhaustive review about the topic of protein labeling, the reader is referred to Marks and Nolan [74].

4. Lateral organization in model membranes

In order to dissect the complexity of actual cell membranes, in which a huge variety of lipids and proteins interact among each other and with molecules in the cytoplasm or in the external environment, a "divide and conquer" strategy promises to be very useful. Keeping this in mind, researchers try to isolate molecules from complex biological contexts to understand their function in simple model systems under controlled conditions. A variety of model membranes have so far been

developed in order to gain insight into membrane processes. Following this approach, a deeper knowledge about how lipids and proteins interact and how these interactions govern the function of cellular membranes has been obtained. It is straightforward that a simple model cannot provide answers for every biological problem: the accuracy and validity of a model bilayer are, in fact, strictly related to the specific question that is to be answered. To date, several types of model membranes have been developed with different characteristics and purposes [75]. Among them, we mention vesicles, black lipid membranes [76,77], monolayers [78], giant unilamellar vesicles (GUVs) [79] and supported lipid bilayers (SLBs) [80]. The last two models are particularly suitable for FCS investigation [61,81,82] both because of the low curvature, comparable to those of cellular membranes, and the large dimensions, much bigger than the FCS focus. SLBs are highly stable, reproducible and can be investigated at the same time with surface sensitive techniques, like Atomic Force Microscopy (AFM) [62,71] or Total Internal Reflection Fluorescence (TIRF) [83]. Unfortunately, the interaction with the support may influence the dynamic behavior of membrane components, especially for trans-membrane proteins with large cytosolic moieties [84]. On the other hand, GUVs have the advantage of being free-standing bilayers and do not present the support-induced artifacts encountered in the case of SLBs [85]. Note that the question of whether a free-standing membrane might be the best approximation for the plasma membrane is not trivial, as it will be discussed in Section 5 of this review. Monitoring the diffusivity of fluorescent lipids, it is possible to gain information about the local viscosity of the bilayer and the molecular interactions among membrane components [63,85]. In the following, we will present recent FCS studies elucidating the topics of lipid–sterol interactions, phase separation in binary and ternary mixtures and, finally, lipid–protein interactions.

Sterol–lipid interactions have been investigated for more than 30 years with a wide variety of biophysical methods [86,87]. Interaction between phospholipids and cholesterol is supposed to result in the formation of a Lo phase, characterized by high degree of both lipid mobility and structural order [88,89]. This specific bilayer phase has increasingly gained popularity as it has been used, also in systems showing coexistence with Ld phase [90,91], to provide a physical model representing the raft-like phase separation present in the cell membranes [15]. Recent studies in our laboratory [85,92] have shown that cholesterol, in mixtures with the low T_m phospholipids DLPC and DOPC, causes a smooth transition from the Ld to the Lo phase, in agreement with the cholesterol-induced "condensing" effect proposed by Demel and de Kruijff [93]. This conclusion was motivated by the monotonous decrease of lipid diffusion coefficients in the fluid bilayer as a function of the increasing cholesterol concentration. Since the decrease in mobility was steeper in the case of DLPC, it was concluded that cholesterol interacts more strongly with saturated phospholipids than unsaturated ones. In the same way, interesting results were obtained making use of high T_m lipids like sphingomyelin (SM) [92], dipalmitoyl-phosphatidylcholine (DPPC) and distearoyl-phosphatidylcholine (DSPC) [94]. In this case, the FCS results suggested that cholesterol disturbs the packing of these lipids, thus increasing the fluidity of the bilayer. No significant difference was observed between the DPPC/cholesterol and the DSPC/cholesterol systems, both less viscous than the bilayers composed of SM and cholesterol. Therefore, it was possible to conclude that: i) the different chain length of DSPC and DPPC does not seem to matter in determining the fluidity of the bilayer and the interactions with the sterol; and ii) SM interacts more effectively with cholesterol, if compared to the other phospholipids examined. These results support the model according to which the lipid–sterol interactions significantly disturb the packing of the bilayer in PC systems, while this perturbation is limited in SM bilayers due to the specific interactions (e.g. strong network of hydrogen bonding) between the sphingolipids and cholesterol [17,87,95–97].

As mentioned above, in order to investigate the physical principles underlying the formation of lipid–protein domains in cell membranes, model bilayers showing phase coexistence were developed [17]. Starting with simple binary mixtures like dilauroyl-phosphatidylcholine (DLPC)/DPPC [81], dioleoyl-phosphatidylcholine (DOPC)/DPPC [71] and dimiristoyl-phosphatidylcholine (DMPC)/DSPC [98], FCS was employed to monitor the diffusion of lipids in the liquid phase, non-ideal mobility and the effects of solid domains as obstacles. The work by Burns et al. [71] put also specific emphasis on the detailed structure of the bilayer in the gel phase and at the interface between domains, thanks also to the high-resolution information provided by AFM. Also, these last three works give the possibility to raise an interesting question, namely the diffusion in gel domains observed via FCS. Burns et al. [71] point out correctly that no diffusion (i.e. slower than the FCS detection limit) would be expected in a gel phase and they do observe, in fact, immobility in such a bilayer. Obviously, this statement depends on the specific technique which is used and the time-scales involved. Interestingly, the other two FCS works cited above [81,98] report diffusion in both gel and liquid phases. These differences could be ascribed to the specific characteristics of the bilayer, like the presence of a ripple phase [98], heterogeneities and packing defects in small gel domains [71]. Nevertheless, it is important to remind the reader about other possible sources of artifacts that might result in similar experimental observations (i.e. a slowly decreasing auto-correlation): bleaching and depletion of the fluorophores [56,71], mechanical instabilities, bright lipid aggregates [63] (i.e. isolated spikes in the signal trace) or local heating.

More complex ternary [92] and quaternary [62] lipid mixtures have been mainly studied to model the raft-like phase separation and, in general, lipid–protein domains in the plasma membrane [17]. More in detail, cholesterol and saturated (sphingo)lipids have been used to form a Lo phase, coexisting with an Ld phase constituted of unsaturated phospholipids. Applying FCS on these model membranes, Kahya et al. [92] have shown that cholesterol is able to modulate lipid dynamics in domains, with the strongest effects in the Lo phase. The study by Scherfeld et al. [94] addressed the role of the sphingosine backbone in the formation of Lo/Ld phase coexistence, applying FCS on mixtures of DPPC/DOPC/cholesterol and DSPC/DOPC/cholesterol. Here, it is argued that the presence of DOPC as third lipid component considerably shifts the delicate balance of lipid–lipid and lipid–sterol interactions. Differently from the already mentioned binary mixtures DPPC/cholesterol and DSPC/cholesterol, the difference in chain length of the palmitoyl and stearoyl acids seems in this case to affect the interaction with cholesterol, inducing large changes in the mobility in the Lo phase [94]. In a similar way, the presence of DOPC and the consequent phase separation enhance the "fluidizing" effect of cholesterol in the Lo phase made of SM [92]. The role of cholesterol in Lo–Ld phase coexistence was also investigated by Bacia et al. [41] by means of methylated β-cyclodextrin (MβCD)-induced cholesterol depletion. The quantitative information provided by FCS allowed a detailed analysis of the hindering of lipid diffusion in the Ld phase, as a consequence of cholesterol removal and Lo domains disappearance. Also, this work offered an example of how FCS can be used to infer the physical state of the bilayer by directly measuring the translational order [17], independently from other methods like the simple observation of fluorescent probe partition [99].

Using the same approach, we used FCS to characterize quantitatively the phase coexistence (i.e. gel, Lo or Ld) in complex mixtures of SM, DOPC, cholesterol and ceramides (Cer) with varying chain length [62]. For example, Cer-rich gel domains [100] were identified on the basis of very low partition and diffusivity of a bulky fluorescent lipid. In two related works, FCS [70] and scanning FCS [23] were applied to similar model membranes with the purpose of investigating the in-plane distribution of cholesterol, as a consequence of long-chain Cer addition. We were thus able to show that Cer inclusion in the Lo phase

triggers the transfer of cholesterol to the Ld phase slowing its dynamics, in agreement with the "cholesterol displacement hypothesis" [101].

The presence of Cer in raft-exhibiting bilayers and in cellular membranes is not only connected to a strong reorganization of the lateral organization of lipids [102,103], but also to that of membrane proteins [32,104]. Since the enrichment of membrane components in Cer domains is associated to dampening of diffusion dynamics [35,62], classic FCS cannot be used to determine the local concentration and the organization of proteins due to strong photobleaching [62]. For this reason, we have used scanning FCS to obtain quantitative information about the partition of membrane proteins between the Ld, Lo and Cer-rich phases [23]. Such precise information could not have been obtained using fluorescence imaging, due to the varying brightness of fluorophores in different membrane environments [99]. FCS is, in fact, not affected by such changes in molecular brightness and can be used to obtain precise partition coefficients [105]. It was thus possible to show that the presence of Cer domains can selectively modulate the lateral distribution of certain membrane proteins and lipids, thus suggesting several mechanisms for the effects of this sphingolipid in vivo [23].

This last example illustrates the advantage of protein reconstitution in model membranes, with the aim of investigating the lipid–protein interplay leading to protein organization in more complex cellular membranes [106,107]. Among the several examples of FCS application for the study of protein dynamics and interaction with model membranes (see e.g. Ref. [108] or [109]), it is also worth citing an interesting characterization of lipid dynamics modulation as a function of protein binding to the membrane surface by Forstner et al. [110] Finally, we would like to mention a considerable portion of FCS studies which have investigated the dynamics and partition in raft-like model membranes of several membrane proteins like SNAREs [23,49], Bacteriorhodopsin [111], GPI-anchored phosphatases [23,63,111] and the beta-secretase BACE [112]. Although not implying a direct identification of Lo domains with rafts in cells, these studies help relating structural features of membrane proteins with their affinity towards ordered or disordered membrane environments and provide information about the thermodynamic stability of lipid–protein interactions [106].

4 1. Asymmetric bilayers

Although most of the biophysical studies regarding model membranes have been performed on bilayers with identical leaflets, it is important to keep in mind that biological membranes are asymmetric [113]. For instance, most of the SM in the plasma membrane resides in the outer leaflet while the phospholipids with serine-, ethanolamine- or inositol-based headgroups are localized mainly in the inner leaflet [114]. Interestingly, the specific lipid mixture constituting the inner leaflet does not seem to produce liquid–liquid phase coexistence [115]. In the last years, several research groups have turned their attention toward the study of model membranes that can take into account the natural asymmetry found in membranes with biological relevance [116–119]. It would be particularly interesting to determine the interaction mechanisms between the two leaflets [120] and, more specifically, how the structural and dynamic properties of the cytosolic leaflet might be influenced by the putative phase separation in the outer leaflet [121].

We would like to point out that, also in this context, FCS could be used to gain information about the detailed structure of the membrane. It was shown, in fact, that FCS measurements can distinguish between the two leaflets using either a selective quenching approach or an asymmetric distribution of the fluorophores [122,123]. Thanks to these methods, Zhang et al. were able to show that both the distal and proximal leaflets of supported membrane systems maintain the same fluidity, even when friction is applied on one leaflet only [123]. Alternatively, fluorescent lipids can be specifically delivered to either the inner or the outer leaflet of the plasma membrane. With this approach, Golebiewska et al. [123b] demonstrated that the lipid phosphatidylinositol 4,5-bisphosphate (PIP(2)) exhibits hindered dynamics only when localized in the cytoplasmic leaflet of plasma membranes, probably due to local reversible binding.

Using methodologies similar to those described above, FCS could also be employed to address the question of leaflet coupling in lipid mixture mimicking the plasma membrane asymmetry.

5. Complex diffusion in cellular membranes

The characterization of diffusion processes in biological systems is crucial to the understanding of the molecular interactions underlying membrane organization. Nevertheless, the information provided by studies in model membranes has always been characterized by a peculiar inconsistency: diffusion in the plasma membrane appears to be orders of magnitude slower than that observed in simple artificial bilayers [27,124]. Furthermore, the motion of membrane components exhibits a deviation from the standard diffusion equation, which would imply a linear growth of the mean square displacement (MSD) with time. In fact, the MSD is often found to vary with a fractional power of time (i.e. $MSD \mu t^{\alpha}$, $0<\alpha<1$) and the exponent α is called anomalous exponent [82,125]. This phenomenon, referred to as anomalous diffusion or subdiffusion [126], was observed experimentally in SPT [47,127] and FRAP [128,129] measurements. More in detail, the work by Feder et al. [130] revealed a direct connection between the immobile fraction reported in recovery experiments [131] and the anomaly in lipid diffusive behavior. In the context of the debate regarding the physical origins of anomalous diffusion in the plasma membrane, Nicolau et al. [132] have recently contributed with a study worthy of note. Using Monte Carlo simulations, three different possible sources for anomalous dynamics were analyzed: immobile randomly distributed obstacles [133], raft-like domains [47] and regular networks of immobile obstacles (i.e. cytoskeleton-anchored picket model) [124]. Although not explicitly considering the possibility of direct interactions with the underlying cytoskeleton (i.e. not mediated by membrane components; "membrane-skeleton fence model" for large proteins [27]), this work probes a wide spectrum of possibilities. Nicolau et al. concluded that tightly packed lipid domains and fixed random obstacles, in absence of other interactions, are the mechanisms most likely inducing anomalous diffusion and values of α significantly lower than unity. For these reasons, it appears clear that free-standing model membranes might not be the best choice to gain further insight into the complex dynamics characterizing the cellular membranes. Rather, direct in vivo measurements would be more appropriate but, unfortunately, the precise evaluation of the anomalous diffusion parameter α by means of FCS measurements on cellular membranes might be challenging [82]. Although more complex mathematical analysis may result very useful [134], the sub-optimal experimental conditions often encountered in cellular measurements do not allow ruling out simple Brownian diffusion models [7]. For this reason, it is possible to find in the scientific literature sound works reporting a broad variety of diffusive behavior for plasma membrane components, ranging from anomalous diffusion of Golgi resident membrane proteins [134] or myelin oligodendrocyte glycoproteins [135] to simple Brownian diffusion of lipids [41,136], integral membrane proteins [55] and GPI-anchored proteins [56].

A very promising alternative approach that uses FCS to probe the details of membrane organization is that proposed by Wawrezinieck et al [137]. Monitoring the change in diffusion time τ of fluorescent lipids or proteins as function of FCS detection area A, it is possible to characterize the submiscroscopical heterogeneities of the bilayer. For free Brownian diffusion, the diffusion time is simply proportional to the detection area ($\tau \propto A$) while, for hindered diffusion with obstacles

much smaller than optical resolution, an offset τ_0 is needed to fit the experimental data points

$$\tau = \text{Const} \cdot A + \tau_0. \quad (5)$$

Whether this offset is positive or negative depends on the exact diffusion mode: dynamic partition into microdomains results in positive τ_0 values, while confinement in cytoskeleton-dependent meshworks results in negative τ_0 values. The size of the detection area A can be varied either using a diaphragm or by means of focusing on different z-positions around the plasma membrane [138]. Exceptional reduction of the detection volume, needed to characterize quantitatively the size of membrane heterogeneities, can be achieved using zero mode waveguides [139]. By means of this approach, Lenne et al. [140] succeeded to determine the diffusive behavior of several membrane components, like GPI-anchored proteins or the Transferrin receptor, elucidating the role of both lipid domains and cytoskeleton-mediated meshwork.

6. Conclusions

In this review, we have provided an overview about FCS and its application for the study of biological membranes. This powerful technique has been effectively used in the last years to investigate lipid–lipid and lipid–protein interactions in model systems. Nevertheless, such sensitive spectroscopic measurements on lipid bilayers are prone to several experimental difficulties. Therefore, a good knowledge of the possible artifact sources is very important for a reliable interpretation of the results. Recent technical developments have also allowed successful application of FCS to more complex systems, like cellular membranes. In this case, calibration-free FCS seems to be the method of choice to obtain quantitative information about lipid and protein dynamics, also in problematic samples. Investigation of diffusion and concentration of several membrane components in complex lipid mixtures is still helping to extend our understanding of the physical principles behind lipid–protein phase separation. Finally, direct measurements on cell membrane are starting now to be successfully applied for the study of the so-called “diffusion laws”. Thanks also to this approach, FCS will likely contribute in the near future to a more complete understanding of lateral organization of cellular membranes.

Acknowledgments

We thank D. Carrer for help with the manuscript and the “Max Planck Gesellschaft” for financial support.

References

[1] D. Magde, E. Elson, W.W. Webb, Thermodynamic fluctuations in a reacting system—measurement by fluorescence correlation spectroscopy, Phys. Rev. Lett. 29 (1972) 705–708.
[2] R. Rigler, U. Mets, J. Widengren, P. Kask, Fluorescence correlation spectroscopy with high count rate and low-background — analysis of translational diffusion, Eur. Biophys. J. Biophys. Lett. 22 (1993) 169–175.
[3] E. Petrov, P. Schwille, State of the art and novel trends in fluorescence correlation spectroscopy, in: U. Resch-Genger (Ed.), Standardization and Quality Assurance in Fluorescence Measurements II: Bioanalytical and Biomedical Applications, Springer, Berlin Heidelberg New York, 2008.
[4] S.A. Kim, K.G. Heinze, P. Schwille, Fluorescence correlation spectroscopy in living cells, Nature Methods 4 (2007) 963–973.
[5] K. Bacia, S.A. Kim, P. Schwille, Fluorescence cross-correlation spectroscopy in living cells, Nat. Methods 3 (2006) 83–89.
[6] R. Rigler, E. Elson, Fluorescence Correlation Spectroscopy: Theory and Applications, Springer, Berlin, 2001.
[7] J. Ries, P. Schwille, New concepts for fluorescence correlation spectroscopy on membranes, PCCP 10 (2008) 3487–3497.
[8] R.B. Gennis, Biomembranes. Molecular structure and function, Springer-Verlag, New York, 1989.
[9] S.J. Singer, G.L. Nicolson, Fluid mosaic model of structure of cell-membranes, Science 175 (1972) 720.
[10] D. Allan, Mapping the lipid distribution in the membranes of BHK cells (mini-review), Mol. Membr. Biol. 13 (1996) 81–84.
[11] E. Rodriguezboulan, W.J. Nelson, Morphogenesis of the polarized epithelial-cell phenotype, Science 245 (1989) 718–725.
[12] D. Tran, J.L. Carpentier, F. Sawano, P. Gorden, L. Orci, Ligands internalized through coated or noncoated invaginations follow a common intracellular pathway, Proc. Nat. Acad. Sci. U. S. A. 84 (1987) 7957–7961.
[13] S. Spiegel, S. Kassis, M. Wilchek, P.H. Fishman, Direct visualization of redistribution and capping of fluorescent gangliosides on lymphocytes, J. Cell Biol. 99 (1984) 1575–1581.
[14] T. Harder, P. Scheiffele, P. Verkade, K. Simons, Lipid domain structure of the plasma membrane revealed by patching of membrane components, J. Cell Biol. 141 (1998) 929–942.
[15] D.A. Brown, E. London, Functions of lipid rafts in biological membranes, Annu. Rev. Cell Dev. Biol. 14 (1998) 111–136.
[16] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.
[17] K. Simons, W.L. Vaz, Model systems, lipid rafts, and cell membranes, Annu. Rev. Biophys. Biomol. Struct. 33 (2004) 269–295.
[18] S. Munro, Lipid rafts: elusive or illusive? Cell 115 (2003) 377–388.
[19] D. Lichtenberg, F.M. Goni, H. Heerklotz, Detergent-resistant membranes should not be identified with membrane rafts, Trends Biochem. Sci. 30 (2005) 430–436.
[20] P.E. Milhiet, C. Domec, M.C. Giocondi, N. Van Mau, F. Heitz, C. Le Grimellec, Domain formation in models of the renal brush border membrane outer leaflet, Biophys. J. 81 (2001) 547–555.
[21] I. Plasencia, L. Norlen, L.A. Bagatolli, Direct visualization of lipid domains in human skin stratum corneum's lipid membranes: effect of pH and temperature, Biophys. J. 93 (2007) 3142–3155.
[22] G. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nat. Rev. 9 (2008) 112–124.
[23] S. Chiantia, J. Ries, G. Chwastek, D. Carrer, Z. Li, R. Bittman, P. Schwille, Role of ceramide in membrane protein organization investigated by combined AFM and FCS, Biochim. Biophys. Acta-Biomembranes 1778 (2008) 1356–1364.
[24] E. Gulbins, H. Grassme, Ceramide and cell death receptor clustering, Biochim. Biophys. Acta 1585 (2002) 139–145.
[25] F. Lafont, S. Lecat, P. Verkade, K. Simons, Annexin XIIIb associates with lipid microdomains to function in apical delivery, J. Cell Biol. 142 (1998) 1413–1427.
[26] K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brugger, M. Simons, Ceramide triggers budding of exosome vesicles into multivesicular Endosomes, Science 319 (2008) 1244–1247.
[27] A. Kusumi, C. Nakada, K. Ritchie, K. Murase, K. Suzuki, H. Murakoshi, R.S. Kasai, J. Kondo, T. Fujiwara, Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules, Annu. Rev. Biophys. Biomol. Struct. 34 (2005) 351–378.
[28] M. Edidin, The state of lipid rafts: from model membranes to cells, Ann. Rev. Biophys. Biomolec. Struct. 32 (2003) 257–283.
[29] B. Storrie, T. Nilsson, The Golgi apparatus: balancing new with old, Traffic 3 (2002) 521–529.
[30] K. Simons, D. Toomre, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell Biol. 1 (2000) 31–39.
[31] K. Simons, R. Ehehalt, Cholesterol, lipid rafts, and disease, J. Clin. Invest. 110 (2002) 597–603.
[32] H. Grassme, H. Schwarz, E. Gulbins, Molecular mechanisms of ceramide-mediated CD95 clustering, Biochem. Biophys. Res. Commun. 284 (2001) 1016–1030.
[33] M.A. del Pozo, N.B. Alderson, W.B. Kiosses, H.H. Chiang, R.G. Anderson, M.A. Schwartz, Integrins regulate Rac targeting by internalization of membrane domains, Science 303 (2004) 839–842.
[34] J.A. Rotolo, J. Zhang, M. Donepudi, H. Lee, Z. Fuks, R. Kolesnick, Caspase-dependent and -independent activation of acid sphingomyelinase signaling, J. Biol. Chem. 280 (2005) 26425–26434.
[35] C.M. Finnegan, S.S. Rawat, E.H. Cho, D.L. Guiffre, S. Lockett, A.H. Merrill, R. Blumenthal, Sphingomyelinase restricts the lateral diffusion of CD4 and inhibits human immunodeficiency virus fusion, J. Virol. 81 (2007) 5294–5304.
[36] A. Gidwani, H.A. Brown, D. Holowka, B. Baird, Disruption of lipid order by short-chain ceramides correlates with inhibition of phospholipase D and downstream signaling by FcepsilonRI, J. Cell Sci. 116 (2003) 3177–3187.
[37] C. Nakada, K. Ritchie, Y. Oba, M. Nakamura, Y. Hotta, R. Iino, R.S. Kasai, K. Yamaguchi, T. Fujiwara, A. Kusumi, Accumulation of anchored proteins forms membrane diffusion barriers during neuronal polarization, Nat. Cell Biol. 5 (2003) 626–632.
[38] H. Murakoshi, R. Iino, T. Kobayashi, T. Fujiwara, C. Ohshima, A. Yoshimura, A. Kusumi, Single-molecule imaging analysis of Ras activation in living cells, Proc. Nat. Acad. Sci. U. S. A. 101 (2004) 7317–7322.
[39] E.A.J. Reits, J.J. Neefjes, From fixed to FRAP: measuring protein mobility and activity in living cells, Nat. Cell Biol. 3 (2001) E145–E147.
[40] D. Axelrod, D.E. Koppel, J. Schlessinger, E. Elson, W.W. Webb, Mobility measurement by analysis of fluorescence photobleaching recovery kinetics, Biophys. J. 16 (1976) 1055–1069.
[41] K. Bacia, D. Scherfeld, N. Kahya, P. Schwille, Fluorescence correlation spectroscopy relates rafts in model and native membranes, Biophys. J. 87 (2004) 1034–1043.
[42] K. Bacia, P. Schwille, A dynamic view of cellular processes by in vivo fluorescence auto- and cross-correlation spectroscopy, Methods 29 (2003) 74–85.

[43] Y.M. Umemura, T.K. Fujiwara, K.G.N. Suzuki, M. Vrljic, S.Y. Nishimura, W.E. Moerner, A. Kusumi, Both MHC class II and its GPI-anchored form undergo hop diffusion as observed by single-molecule tracking, Biophys. J. (2007) 527A.
[44] G.J. Schutz, M. Sonnleitner, P. Hinterdorfer, H. Schindler, Single molecule microscopy of biomembranes (review), Mol. Membr. Biol. 17 (2000) 17–29.
[45] G.J. Schütz, P. Hinterdorfer, Single molecule fluorescence and force microscopy, Exp. Gerontol. 37 (2002) 1495–1511.
[46] T. Ha, Single-molecule fluorescence methods for the study of nucleic acids, Curr. Opin. Struct. Biol. 11 (2001) 287–292.
[47] G.J. Schutz, H. Schindler, T. Schmidt, Single-molecule microscopy on model membranes reveals anomalous diffusion, Biophys. J. 73 (1997) 1073–1080.
[48] L. Guo, J.Y. Har, J. Sankaran, Y.M. Hong, B. Kannan, T. Wohland, Molecular diffusion measurement in lipid bilayers over wide concentration ranges: a comparative study, Chemphyschem 9 (2008) 721–728.
[49] K. Bacia, C.G. Schuette, N. Kahya, R. Jahn, P. Schwille, SNAREs prefer liquid-disordered over "raft" (liquid-ordered) domains when reconstituted into giant unilamellar vesicles, J. Biol. Chem 279 (2004) 37951–37955.
[50] P. Liu, T. Sudhaharan, R.M.L. Koh, L.C. Hwang, S. Ahmed, I.N. Maruyama, T. Wohland, Investigation of the dimerization of proteins from the epidermal growth factor receptor family by single wavelength fluorescence cross-correlation spectroscopy, Biophys. J. 93 (2007) 684–698.
[51] D.R. Larson, J.A. Gosse, D.A. Holowka, B.A. Baird, W.W. Webb, Temporally resolved interactions between antigen-stimulated IgE receptors and Lyn kinase on living cells, J. Cell Biol. 171 (2005) 527–536.
[52] K. Bacia, I.V. Majoul, P. Schwille, Probing the endocytic pathway in live cells using dual-color fluorescence cross-correlation analysis, Biophys. J. 83 (2002) 1184–1193.
[53] K. Bacia, P. Schwille, Practical guidelines for dual-color fluorescence cross-correlation spectroscopy, Nat. Protoc. 2 (2007) 2842–2856.
[54] J. Enderlein, I. Gregor, D. Patra, T. Dertinger, U.B. Kaupp, Performance of fluorescence correlation spectroscopy for measuring diffusion and concentration, Chemphyschem 6 (2005) 2324–2336.
[55] J. Ries, P. Schwille, Studying slow membrane dynamics with continuous wave scanning fluorescence correlation spectroscopy, Biophys. J. 91 (2006) 1915–1924.
[56] J. Ries, S. Chiantia, P. Schwille, Accurate determination of membrane dynamics with line scan FCS. Biophys. J. submitted for publication
[57] B. Hebert, S. Costantino, P.W. Wiseman, Spatiotemporal image correlation spectroscopy (STICS) theory, verification, and application to protein velocity mapping in living CHO cells, Biophys. J. 88 (2005) 3601–3614.
[58] A.G. Ayuyan, F.S. Cohen, Lipid peroxides promote large rafts: effects of excitation of probes in fluorescence microscopy and electrochemical reactions during vesicle formation, Biophys. J. 91 (2006) 2172–2183.
[59] N.L. Thompson, B.L. Steele, Total internal reflection with fluorescence correlation spectroscopy, Nat. Protoc. 2 (2007) 878–890.
[60] J. Ries, T. Ruckstuhl, D. Verdes, P. Schwille, Supercritical angle fluorescence correlation spectroscopy, Biophys. J. 94 (2008) 221–229.
[61] A. Benda, M. Benes, V. Marecek, A. Lhotsky, W.T. Hermens, M. Hof, How to determine diffusion coefficients in planar phospholipid systems by confocal fluorescence correlation spectroscopy, Langmuir 19 (2003) 4120–4126.
[62] S. Chiantia, N. Kahya, P. Schwille, Raft domain reorganization driven by short- and long-chain ceramide: a combined AFM and FCS study, Langmuir 23 (2007) 7659–7665.
[63] S. Chiantia, J. Ries, N. Kahya, P. Schwille, Combined AFM and two-focus SFCS study of raft-exhibiting model membranes, Chemphyschem 7 (2006) 2409–2418.
[64] T. Dertinger, I. der Hocht, A. Benda, M. Hof, J. Enderlein, Surface sticking and lateral diffusion of lipids in supported bilayers, Langmuir 22 (2006) 9339–9344.
[65] Q.Q. Ruan, M.A. Cheng, M. Levi, E. Gratton, W.W. Mantulin, Spatial–temporal studies of membrane dynamics: scanning fluorescence correlation spectroscopy (SFCS), Biophys. J. 87 (2004) 1260–1267.
[66] Z. Petrasek, P. Schwille, Precise measurement of diffusion coefficients using scanning fluorescence correlation spectroscopy, Biophys. J. 94 (2008) 1437–1448.
[67] T. Dertinger, V. Pacheco, I. der Hocht, R. Hartmann, I. Gregor, J. Enderlein, Two-focus fluorescence correlation spectroscopy: a new tool for accurate and absolute diffusion measurements, Chemphyschem 8 (2007) 433–443.
[68] M. Burkhardt, P. Schwille, Electron multiplying CCD based detection for spatially resolved fluorescence correlation spectroscopy, Opt. Express 14 (2006) 5013–5020.
[69] A.J. Garcia-Saez, P. Schwille, Fluorescence correlation spectroscopy for the study of membrane dynamics and protein/lipid interactions, Methods (2008), doi:10.1016/j.ymeth.2008.06.011.
[70] S. Chiantia, N. Kahya, J. Ries, P. Schwille, Effects of ceramide on liquid-ordered domains investigated by simultaneous AFM and FCS, Biophys. J. 90 (2006) 4500–4508.
[71] A.R. Burns, D.J. Frankel, T. Buranda, Local mobility in lipid domains of supported bilayers characterized by atomic force microscopy and fluorescence correlation spectroscopy, Biophys. J. 89 (2005) 1081–1093.
[72] Z.G. Li, E. Mintzer, R. Bittman, First synthesis of free cholesterol — BODIPY conjugates, J. Org. Chem. 71 (2006) 1718–1721.
[73] J.E. Shaw, R.F. Epand, R.M. Epand, Z.G. Li, R. Bittman, C.M. Yip, Correlated fluorescence-atomic force microscopy of membrane domains: structure of fluorescence probes determines lipid localization, Biophys. J. 90 (2006) 2170–2178.
[74] K.M. Marks, G.P. Nolan, Chemical labeling strategies for cell biology, Nat. Methods 3 (2006) 591–596.
[75] E. Sackmann, Supported membranes: scientific and practical applications, Science 271 (1996) 43–48.
[76] M.K. Jain, The bimolecular lipid membrane: a system, Litton Educational Publ., New York, 1972, pp. 52–144.
[77] P.F. Fahey, D.E. Koppel, L.S. Barak, D.E. Wolf, E.L. Elson, W.W. Webb, Lateral diffusion in planar lipid bilayers, Science 195 (1977) 305–306.
[78] M.M. Lipp, K.Y.C. Lee, J.A. Zasadzinski, A.J. Waring, Phase and morphology changes in lipid monolayers induced by SP-B protein and its amino-terminal peptide, Science 273 (1996) 1196–1199.
[79] F.M. Menger, J.S. Keiper, Chemistry and physics of plant vesicles as biomembrane models, Curr. Opin. Chem. Biol. 2 (1998) 726–732.
[80] V. Vontscharner, H.M. Mcconnell, An alternative view of phospholipid phase-behavior at the air–water-interface — microscope and film balance studies, Biophys. J. 36 (1981) 409–419.
[81] J. Korlach, P. Schwille, W.W. Webb, G.W. Feigenson, Characterization of lipid bilayer phases by confocal microscopy and fluorescence correlation spectroscopy, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 8461–8466.
[82] P. Schwille, J. Korlach, W.W. Webb, Fluorescence correlation spectroscopy with single-molecule sensitivity on cell and model membranes, Cytometry 36 (1999) 176–182.
[83] L. Guo, J.Y. Har, J. Sankaran, Y.M. Hong, B. Kannan, T. Wohland, Molecular diffusion measurement in lipid bilayers over wide concentration ranges: a comparative study, Chemphyschem 9 (2008) 721–728.
[84] A.J. Diaz, F. Albertorio, S. Daniel, P.S. Cremer, Double cushions preserve transmembrane protein mobility in supported bilayer systems, Langmuir 24 (2008) 6820–6826.
[85] N. Kahya, D. Scherfeld, K. Bacia, P. Schwille, Lipid domain formation and dynamics in giant unilamellar vesicles explored by fluorescence correlation spectroscopy, J. Struct. Biol. 147 (2004) 77–89.
[86] H.M. McConnell, A. Radhakrishnan, Condensed complexes of cholesterol and phospholipids, Biochim. Biophys. Acta-Biomembranes 1610 (2003) 159–173.
[87] B. Ramstedt, J.P. Slotte, Sphingolipids and the formation of sterol-enriched ordered membrane domains, Biochim. Biophys. Acta-Biomembranes 1758 (2006) 1945–1956.
[88] J.H. Ipsen, G. Karlstrom, O.G. Mouritsen, H. Wennerstrom, M.J. Zuckermann, Phase-equilibria in the phosphatidylcholine–cholesterol system, Biochim. Biophys. Acta 905 (1987) 162–172.
[89] M.R. Vist, J.H. Davis, Phase-equilibria of cholesterol dipalmitoylphosphatidylcholine mixtures — H-2 nuclear magnetic-resonance and differential scanning calorimetry, Biochemistry 29 (1990) 451–464.
[90] M.B. Sankaram, T.E. Thompson, Cholesterol-induced fluid-phase immiscibility in membranes, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 8686–8690.
[91] A.T. Hammond, F.A. Heberle, T. Baumgart, D. Holowka, B. Baird, G.W. Feigenson, Crosslinking a lipid raft component triggers liquid ordered-liquid disordered phase separation in model plasma membranes, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 6320–6325.
[92] N. Kahya, D. Scherfeld, K. Bacia, B. Poolman, P. Schwille, Probing lipid mobility of raft-exhibiting model membranes by fluorescence correlation spectroscopy, J. Biol. Chem. 278 (2003) 28109–28115.
[93] R.A. Demel, B. Dekruyff, Function of sterols in membranes, Biochim. Biophys. Acta 457 (1976) 109–132.
[94] D. Scherfeld, N. Kahya, P. Schwille, Lipid dynamics and domain formation in model membranes composed of ternary mixtures of unsaturated and saturated phosphatidylcholines and cholesterol, Biophys. J. 85 (2003) 3758–3768.
[95] H. Ohvo-Rekila, B. Ramstedt, P. Leppimaki, J.P. Slotte, Cholesterol interactions with phospholipids in membranes, Prog. Lipid Res. 41 (2002) 66–97.
[96] T. Rog, M. Pasenkiewicz-Gierula, Cholesterol–sphingomyelin interactions: a molecular dynamics simulation study, Biophys. J. 91 (2006) 3756–3767.
[97] P.R. Maulik, G.G. Shipley, Interactions of *N*-stearoyl sphingomyelin with cholesterol and dipalmitoyl phosphatidylcholine in bilayer membranes, Biophys. J. 70 (1996) 2256–2265.
[98] A.E. Hac, H.M. Seeger, M. Fidorra, T. Heimburg, Diffusion in two-component lipid membranes — a fluorescence correlation spectroscopy and Monte Carlo simulation study, Biophys. J. 88 (2005) 317–333.
[99] T. Baumgart, G. Hunt, E.R. Farkas, W.W. Webb, G.W. Feigenson, Fluorescence probe partitioning between L-o/L-d phases in lipid membranes, Biochim. Biophys. Acta-Biomembranes 1768 (2007) 2182–2194.
[100] C.R. Bollinger, V. Teichgraber, E. Gulbins, Ceramide-enriched membrane domains, Biochim. Biophys. Acta 1746 (2005) 284–294.
[101] Megha, E. London, Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function, J. Biol. Chem. 279 (2004) 9997–10004.
[102] A.E. Cremesti, F.M. Goni, R. Kolesnick, Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outcome? FEBS Lett. 531 (2002) 47–53.
[103] I. Johnston, L.J. Johnston, Ceramide promotes restructuring of model raft membranes, Langmuir 22 (2006) 11284–11289.
[104] H. Grassme, V. Jendrossek, J. Bock, A. Riehle, E. Gulbins, Ceramide-rich membrane rafts mediate CD40 clustering, J. Immunol. 168 (2002) 298–307.
[105] L. Rajendran, A. Schneider, G. Schlechtingen, S. Weidlich, J. Ries, T. Braxmeier, P. Schwille, J.B. Schulz, C. Schroeder, M. Simons, G. Jennings, H.J. Knolker, K. Simons, Efficient inhibition of the Alzheimer's disease beta-secretase by membrane targeting, Science 320 (2008) 520–523.
[106] N. Kahya, Targeting membrane proteins to liquid-ordered phases: molecular self-organization explored by fluorescence correlation spectroscopy, Chem. Phys. Lipids. 141 (2006) 158–168.

[107] S. Mayor, M. Rao, Rafts: scale-dependent, active lipid organization at the cell surface, Traffic 5 (2004) 231–240.
[108] M.K. Doeven, J.H.A. Folgering, V. Krasnikov, E.R. Geertsma, G. van den Bogaart, B. Poolman, Distribution, lateral mobility and function of membrane proteins incorporated into giant unilamellar vesicles, Biophys. J. 88 (2005) 1134–1142.
[109] A. Miszta, R. Machan, A. Benda, A.J. Ouellette, W.T. Hermens, M. Hof, Combination of ellipsometry, laser scanning microscopy and Z-scan fluorescence correlation spectroscopy elucidating interaction of cryptdin-4 with supported phospholipid bilayers, J. Pept. Sci. 14 (2008) 503–509.
[110] M.B. Forstner, C.K. Yee, A.N. Parikh, J.T. Groves, Lipid lateral mobility and membrane phase structure modulation by protein binding, J. Am. Chem. Soc. 128 (2006) 15221–15227.
[111] N. Kahya, D.A. Brown, P. Schwille, Raft partitioning and dynamic behavior of human placental alkaline phosphatase in giant unilamellar vesicles, Biochemistry 44 (2005) 7479–7489.
[112] L. Kalvodova, N. Kahya, P. Schwille, R. Ehehalt, P. Verkade, D. Drechsel, K. Simons, Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids in vitro, J. Biol. Chem. 280 (2005) 36815–36823.
[113] M.S. Bretsche, Asymmetrical lipid bilayer structure for biological-membranes, Nat.-New Biol. 236 (1972) 11–12.
[114] P.F. Devaux, Static and dynamic lipid asymmetry in cell-membranes, Biochemistry 30 (1991) 1163–1173.
[115] T.Y. Wang, J.R. Silvius, Cholesterol does not induce segregation of liquid-ordered domains in bilayers modeling the inner leaflet of the plasma membrane, Biophys. J. 81 (2001) 2762–2773.
[116] J.M. Crane, V. Kiessling, L.K. Tamm, Measuring lipid asymmetry in planar supported bilayers by fluorescence interference contrast microscopy, Langmuir 21 (2005) 1377–1388.
[117] E. Kalb, S. Frey, L.K. Tamm, Formation of supported planar bilayers by fusion of vesicles to supported phospholipid monolayers, Biochim. Biophys. Acta 1103 (1992) 307–316.
[118] M.D. Collins, S.L. Keller, Direct observation of phospholipid flip-flop in an unsupported, asymmetric model lipid bilayer, Biophys. J. (2007) 15A.
[119] A.J. Wagner, S. Loew, S. May, Influence of monolayer–monolayer coupling on the phase behavior of a fluid lipid bilayer, Biophys. J. 93 (2007) 4268–4277.
[120] M.D. Collins, Interleaflet coupling mechanisms in bilayers of lipids and cholesterol, Biophys. J. 94 (2008) L32–L34.
[121] V. Kiessling, J.M. Crane, L.K. Tamm, Transbilayer effects of raft-like lipid domains in asymmetric planar bilayers measured by single molecule tracking, Biophys. J. 91 (2006) 3313–3326.
[122] L.F. Zhang, S. Granick, Lipid diffusion compared in outer and inner leaflets of planar supported bilayers, J. Chem. Phys. 123 (2005).
[123] L.F. Zhang, S. Granick, Interleaflet diffusion coupling when polymer adsorbs onto one sole leaflet of a supported phospholipid bilayer, Macromolecules 40 (2007) 1366–1368.
[123b] U. Golebiewska, M. Nyako, W. Woturski, I. Zaitseva, S. Mc Laughlin, Diffusion coefficent of fluorescent phosphatidylinositol 4,5-biphosphate in the plasma membrane of cells, Mol. Biol. Cell 19 (2008) 1663–1669.
[124] T. Fujiwara, K. Ritchie, H. Murakoshi, K. Jacobson, A. Kusumi, Phospholipids undergo hop diffusion in compartmentalized cell membrane, J. Cell Biol. 157 (2002) 1071–1081.
[125] J.P. Bouchaud, A. Georges, Anomalous diffusion in disordered media — statistical mechanisms, models and physical applications, Phys. Rep.-Rev. Sect. Phys. Lett. 195 (1990) 127–293.
[126] M.J. Saxton, Anomalous diffusion due to obstacles — a Monte-Carlo study, Biophys. J. 66 (1994) 394–401.
[127] A. Kusumi, H. Ike, C. Nakada, K. Murase, T. Fujiwara, Single-molecule tracking of membrane molecules: plasma membrane compartmentalization and dynamic assembly of raft-philic signaling molecules, Semin. Immunol. 17 (2005) 3–21.
[128] I. BrustMascher, T.J. Feder, J.P. Slattery, B. Baird, W.W. Webb, FPR data on mobility of cell-surface proteins reevaluated in terms of temporally constrained molecular motions, Biophys. J. 64 (1993) A354.
[129] A.K. Kenworthy, B.J. Nichols, C.L. Remmert, G.M. Hendrix, M. Kumar, J. Zimmerberg, J. Lippincott-Schwartz, Dynamics of putative raft-associated proteins at the cell surface, J. Cell Biol. 165 (2004) 735–746.
[130] T.J. Feder, I. BrustMascher, J.P. Slattery, B. Baird, W.W. Webb, Constrained diffusion or immobile fraction on cell surfaces: a new interpretation, Biophys. J. 70 (1996) 2767–2773.
[131] L.K. Tamm, Lateral diffusion and fluorescence microscope studies on a monoclonal-antibody specifically bound to supported phospholipid-bilayers, Biochemistry 27 (1988) 1450–1457.
[132] D.V. Nicolau, J. Hancock, K. Burrage, Sources of anomalous diffusion on cell membranes: a Monte Carlo study, Biophys. J. 92 (2008) 1975–1987.
[133] H. Berry, Monte Carlo simulations of enzyme reactions in two dimensions: fractal kinetics and spatial segregation, Biophys. J. 83 (2002) 1891–1901.
[134] M. Weiss, H. Hashimoto, T. Nilsson, Anomalous protein diffusion in living cells as seen by fluorescence correlation spectroscopy, Biophys. J. 84 (2003) 4043–4052.
[135] E. Gielen, J. Vercammen, J. Sykora, J. Humpolickova, M. vandeVen, A. Benda, N. Hellings, M. Hof, Y. Engelborghs, P. Steels, M. Ameloot, Diffusion of sphingomyelin and myelin oligodendrocyte glycoprotein in the membrane of OLN-93 oligodendroglial cells studied by fluorescence correlation spectroscopy, C. R., Biol. 328 (2005) 1057–1064.
[136] I. Gombos, G.B. Steinbach, I. Pomozi, A. Balogh, G. Vamosi, A. Gansen, G. Laszlo, G. Garab, J. Matko, Some new faces of membrane microdomains: a complex confocal fluorescence, differential polarization, and FCS imaging study on live immune cells, Cytometry Part A 73A (2008) 220–229.
[137] L. Wawrezinieck, H. Rigneault, D. Marguet, P.F. Lenne, Fluorescence correlation spectroscopy diffusion laws to probe the submicron cell membrane organization, Biophys. J. 89 (2005) 4029–4042.
[138] J. Humpolickova, E. Gielen, A. Benda, V. Fagulova, J. Vercammen, M. Vandeven, M. Hof, M. Ameloot, Y. Engelborghs, Probing diffusion laws within cellular membranes by Z-scan fluorescence correlation spectroscopy, Biophys. J. 91 (2006) L23–L25.
[139] J. Wenger, F. Conchonaud, J. Dintinger, L. Wawrezinieck, T.W. Ebbesen, H. Rigneault, D. Marguet, P.F. Lenne, Diffusion analysis within single nanometric apertures reveals the ultrafine cell membrane organization, Biophys. J. 92 (2007) 913–919.
[140] P.F. Lenne, L. Wawrezinieck, F. Conchonaud, O. Wurtz, A. Boned, X.J. Guo, H. Rigneault, H.T. He, D. Marguet, Dynamic molecular confinement in the plasma membrane by microdomains and the cytoskeleton meshwork, Embo J. 25 (2006) 3245–3256.