Chapter 1 Introduction

1.1 Objectives and outlines

Fluorescence microscopy is a fast growing branch of optical microscopy, a branch the applications of which lie mainly in the life science research. This work focuses on one special method of fluorescence microscopy, on Fluorescence Correlation Spectroscopy (FCS). The major aim of this work is to develop and demonstrate new possibilities offered by this popular method.

The presented work can be understood from three levels. The first level are the concepts. Firstly, it brings a methodology how to precisely measure diffusion coefficients of labeled species in planar systems using confocal FCS. Secondly, it shows a way for overcoming problems of multiple species analysis using the time-resolved detection. Thirdly, it joins the preceding two concepts and introduces a novel approach how to simultaneously follow and resolve the surface confined and free space diffusion.

The second level is the least visible, but probably the most laborious one. It is about the hidden background of hardware upgrades, improved ways of sample preparation and manipulation, and faster and more robust data analysis including new software algorithms, programs and macros with user friendly interfaces. In other words, the second level are the tools that enable to realize the concepts.

The third level are the applications that arose from the new concepts utilizing the developed tools. The applications reported in this work are mainly from the field of lipid membranes, protein-membrane interactions and DNA condensation.

Chapter 1 gives a brief overview of the main methods and model system used in this thesis. Chapter 2 is based on Paper I, on the "Z-scan" method for precise determination of diffusion coefficients in planar systems, and its various extensions and applications. The hardware and software implementation of Time-Resolved Fluorescence Correlation Spectroscopy (Paper II) together with the resulting applications is described in Chapter 3. The last Chapter 4 is devoted to the fluorescence lifetime tuning within supported phospholipids bilayers, which combined with Time-Resolved Fluorescence Correlation Spectroscopy enables to simultaneously follow and resolve the supported phospholipids bilayers confined and free bulk diffusion (Paper III).

1.2 Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) was invented in the early 70s by the group of Prof. Webb[1]. It relies on the statistical correlation analysis of fluctuations in the fluorescence signal coming from a small number (ideally units) of fluorescent molecules. It gives us information on processes that lead to these intensity fluctuations. The processes are mainly translational[2] and rotational[3] diffusion, flow[4], conformational fluctuations[5], chemical reactions[6] or photophysical excited state processes of the dye[7]. It started to flourish in the 90s by the introduction of advanced confocal microscopes, stable lasers, fast sensitive detectors and fast electronics[8]. The technical development led to a huge improvement in S/N ratio and thus increased the sensitivity down to a single molecule level. Nowadays it is widely accepted as a standard method for measuring diffusion coefficients and low concentrations of fluorescent molecules in vitro and combined with a confocal scanning also in vivo[9].

1.3 Time-Resolved Fluorescence Correlation Spectroscopy

Standard FCS had soon reached its limits[10] and new ways to enhance its capabilities were searched for. The task was to simultaneously monitor diffusion and concentration of two or more labeled species, and to discover if there is any interaction between them. The first solution came in the form of two-color FCS[11], where two coupled lasers and two detectors using different spectral regions are used. A necessary prerequisite is to have two dyes with sufficiently separated spectra. Another solution, the so-called Time-Resolved FCS (TR-FCS)[12], instead of different spectra uses different excited state fluorescence lifetimes to calculate auto- and cross- correlation curves of species with different lifetimes from a mixture. This revolutionary approach is simpler to implement than the two-color FCS setup, but the data analysis is more complicated and requires special data storage and analysis

algorithms. In the case of TR-FCS two fluorescent species with similar spectra, but with sufficiently different excited state lifetimes are required.

1.4 Ellipsometry

Ellipsometry is a very sensitive non-destructive optical technique for the characterization and observation of events at an interface or film between two media. It is based on exploiting the polarization transformation that occurs as a beam of polarized light is reflected from a surface or a film. Ellipsometry is a convenient and accurate technique for the measurement of optical constants of reflecting surfaces and for the measurement of thicknesses and refractive indexes of very thin films on solid surfaces. Moreover, it can also monitor the kinetics of the adsorption process or phase change, and if we know the chemical composition of the adsorbed layer, its surface mass can be calculated as well[13].

First ellipsometry measurements on protein adsorption to surfaces was carried out already in 1942 by Rothen[14], first measurement on supported lipid membranes and subsequent protein absorption were performed in the eighties by the group of Hermens[13, 15].

1.5 Supported Phospholipid Bilayers (SPBs)

Supported Phospholipid Bilayers (SPBs) are a popular model of biomembranes. They consist of planar lipid bilayers attached to a hydrophilic surface. Their main advantages are easy creation, simple manipulation, long term stability and mainly the accessibility for many surface sensitive techniques (AFM, QCM-D, SPR, ellipsometry,...). These properties cause the wide use of SPBs in biosensors, micro- and nano-structures, blood-compatible surfaces, medical implant devices, and production of catalytic interfaces[16]. Many additional applications are proposed or are currently under study[17-19].

SPBs can be created by two main approaches, either by the laborious Langmuir-Blodgett stacking technique, or by exposure of a solid support to phospholipid vesicle suspensions[20]. The latter method is the preferred one as it gives defect free bilayers and is much simpler. Despite their wide use, the mechanisms of

SPBs creation from vesicles and the influence of preparation conditions on the process of creation are not yet totally clear[21].

Chapter 2 Z-scan (Paper I)

2.1 Z-scan

The successful use of FCS in solution led to first FCS trials on planar membranes[22, 23]. However, the precise sample localization respective to the detection volume (laser beam) appeared to be an inherent problem. The orientation and the position of the thin (5 nm) lipid membrane relative to the detection volume (0.5 x 0.5 x 3 μm^3) changes the illuminated area of the membrane and thus hugely influences the resulting autocorrelation functions and parameters derived from it. We showed that the original intensity based approach leads to a rather large systematic error and developed a new methodology for more precise determination of diffusion coefficients in planar membranes[Paper I]. It is based on a series of measurements at different, relative z-positions of the sample and the laser focus, and analyzing the resulting dependencies. The major advantages of the presented Z-scan are:

1. Precise localization of the sample
2. Withdrawal of external calibration of the focal volume size
3. Improved precision of diffusion coefficient determination down to a relative standard deviation of 5%
4. Distinguishing between lateral diffusion and other processes

The Z-scan was applied on SPBs as well as on phospholipids adsorbed at water-dichlorethan interface. The novelty of the Z-scan approach as well as the determination of diffusion coefficients at liquid-liquid interface has to be underlined.

2.2 Comparison of one- and two- photon excitation

The Z-scan method has been since its introduction further studied and optimized. This included the influence of experimental conditions, the pinhole size, the used laser intensity and the mode of excitation, on the Z-scan performance. Particularly interesting was the comparison of Z-scan under one- and two- photon excitation (collaboration with Prof. Y. Mely, Strasbourg, France).

Both modes of excitation showed the Z-scan predicted parabolic dependences, but the diffusion coefficient from the two-photon excitation was 3 times larger than the diffusion coefficient from the one-photon excitation under standard instrumental setup. Later experiments studying the influence of the used laser intensity on the resulting fit parameters revealed a huge impact of photobleaching on the apparent diffusion time; an effect also enhanced by the slowness of the measured diffusion in SPBs. Under conditions, where the effect of photobleaching is minimized and both excitation modes give the same diffusion coefficients, the signal to noise ratio for one-photon excitation is an order of magnitude higher than for two-photon case. These observations can be explained by different mechanisms of photobleaching mechanism for one- and two- photon excitation (from triplet state and multi-photon radical creation, respectively[24]).

2.3 Multi-focus FCS (Paper VIII, Paper IX)

As mentioned in the previous chapters, the diffusion coefficients measured by FCS are prone to many experimental artifacts coming mainly from the uncertainties in the detection volume (illuminated area) size. The measured diffusion time thus cannot be precisely transferred to the diffusion coefficient. To overcome this problem, the so-called multi-focus FCS was suggested by Enderlein and experimentally realized by Dertinger.

There are two key ideas behind the multi-focus FCS. The first idea is to replace the uncertain size of the detection volume used in the standard FCS by another precisely measurable parameter – to introduce two or more partially overlapping laser foci, mutual distances of which are measurable by CCD camera and serve as an exact ruler. The ratio of cross- and auto-correlation functions of the two overlapping foci then contains a term, which is dependant on the distances of the foci and thus gives precise values of diffusion coefficients.

The second idea consists in the high repetition sequential pulsing of the excitation sources that enables to attribute the focus of origin for every detected photon using the time-tagged time resolved (TTTR) data storing.

For testing of this newly designed setup measurements of lipid diffusion in SPBs were performed and directly compared with Z-scan approach[Paper VIII, Paper IX].

After the first testing[Paper VIII], the original hardware realization was slightly improved[Paper IX], leading to the overall improvement of measurement characteristics, giving identical diffusion coefficients of labeled lipid in SPBs for both Z-scan and multi-focus methods.

The multi-focus FCS was shown to be a valuable alternative to the standard one-focus FCS. The advantage is its insensitivity to precise z-positioning, as it depends only on the mutual distance of the laser beams and no Z-scan is thus needed. On the other hand, to obtain good measurement statistics longer measurement times are required, and due to longer cross-correlation times, the times the fluorophore stays illuminated, it is more susceptible to photobleaching artifacts.

2.4 Z-scan on living cells – a way to study confined diffusion (Paper V, Paper X)

After the successful application of Z-scan on model lipid membranes, the next step was to introduce it also to the living cell research. The first measurements were done on living oligodendrocytes, which are the myelin-producing cells of the central nervous system, using Confocor 2 apparatus (collaboration with Prof. Yves Engelborghs, University of Leuven, Belgium).
The original aim was just to improve the cell-membrane FCS measurement quality, which means precise localizing of the membrane, avoiding the external calibration and resolving between membrane and free space diffusion, and by these improvements to enable systematic studies on cells (cell-membrane response to different stimuli)[Paper X]. But apart from this, another very useful feature of Z-scan was discovered.

The plasma membrane of living cells is heterogeneous in structure and may contain microdomains, which can impose constraints on the lateral diffusion of its constituents. The size of these microdomains is under diffraction resolution limit of visible light optical methods. Prieto et al[25-27] use indirect method of Fluorescence Resonance Energy Transfer (FRET) to study the size of the microdomains and their phase behavior on model systems. FCS is used to directly investigate the labeled lipid diffusion in different phases of lipid membranes. When the domains are larger

than the optical resolution, interpretation of the results is rather straightforward[28]. In the case of microdomains, where the signal comes from both phases and the resulting ACF is governed by diffusion in fluid phase, in gel phase and by interchange between these phases, standard FCS measurement gives ACF that can be very well fitted with the standard one component 2D diffusion model. Thus it is impossible to decide whether we have a system with slow diffusion and without microdomains or a system with fast diffusion and with microdomains. Very recently L. Wawrezieck et al[29] 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 show that the Z-scan is an alternative approach which yields essentially the same information and which can readily be applied on commercial FCS instruments[Paper V]. The change of the illuminated area size is not performed by changing the underfilling of the objective, but by simple movement of the focus in z-axis. The measured diffusion time is plotted against the measured particle number, which serves as an indicator of the illuminated area size. The intercept of the linear fit of this dependence with the y-axes (diffusion time axes) tells us about the nature of the confinement. For model system of SPBs it is close to zero, whereas for the cells it is significantly different from zero and positive, suggesting diffusion hindered by rafts.

2.5 Comparison of lipid diffusion in supported and free standing membrane (Paper VI)

A long lasting question in SPBs research is how much is the diffusion of the lipids in SPBs influenced by the presence of the support. A comparison of the diffusion in SPBs with the diffusion in free standing membrane in giant unilamellar vesicles (GUVs) was hampered by differences in experimental conditions used for the preparation of these two model membranes and by the lack of precise tools for diffusion coefficient determination.

In Paper VI we performed an absolute comparison of diffusion coefficients in supported and free standing bilayers using the Z-scan. The experiments were carried out using the new confocal scanning microscope MicroTime 200 (PicoQuant, Germany). The absolute comparison means that all experimental conditions were identical – both membranes were prepared from the same lipid mixture and the measurements were performed in one cuvette, having the buffer composition, temperature and instrumental setup identical.

The diffusion coefficient of β-C8-BODIPY® 500/510 C5-HPC in DOPC bilayers is 7.8 ± 0.8 $\mu m^2 s^{-1}$ for the free standing form (GUVs) and 2.9 ± 0.3 $\mu m^2 s^{-1}$ for the supported form (SPBs) at temperature 16°C.

The observed large difference of lipid mobility in free and supported membranes may by explained by weaker, but still substantial, frictional coupling between the support and the inner leaflet than between the bilayer leaflets[30, 31]. Another possible mechanism for slowing down the lipid diffusion might be the partial restriction of the motion of the lipid molecules in the plane of the bilayer caused by the (electrostatic) interaction with a flat support. As a consequence, lipid molecules partially lose one degree of freedom how to pass by the adjacent molecules, which results in a slower diffusion.

2.6 Combining FCS with ellipsometry (Paper IV, Paper VII)

For the applications of SPBs it is necessary to know that the bilayer is complete and stable. We applied the ellipsometry to study the kinetics of SPBs formation on mica, glass and oxidized silicon[Paper IV]. Using the ellipsometry we followed the kinetics of vesicle adsorption and the way how it depends on the surface, phospholipid composition and presence of Ca^{2+} cations. Different mechanisms of formation were described and the optimal conditions for defects free SPBs preparation were found.

One of the most interesting joint applications of SPBs, Z-scan and ellipsometry is the study of membrane-protein interactions. SPBs serve as an easy to handle model of biomembranes, ellipsometry gives information about the kinetics of

the protein-membrane binding and equilibrium binding constants and FCS tell us how the lipid mobility is influenced by the protein binding[Paper VII] or what the mobility of the fluorescently labeled bound proteins is.

In order to be able to accomplish the combined FCS-ellipsometry measurements, we constructed a combined FCS-ellipsometry cuvette that enables measurement of the same sample on both instruments, giving us a direct comparison of information from both methods.

We use this cuvette for the study of the interaction of antimicrobial peptides with SPBs. First that time purely FCS results about the influence of the protein binding on the mobility of SPBs are presented in Paper VII. We continue in that study using the combined FCS-ellipsometry approach and a new manuscript containing very detailed results comparing the different mechanisms of interaction with lipid membranes for antimicrobial peptides Melittin, Magainin 2 and Cryptdin-4 is being prepared (Interaction of antimicrobial peptides with supported phospholipid bilayers studied by ellipsometry and fluorescence correlation spectroscopy; A. Miszta, A. Benda, A. J. Ouellette, R. Jelinek, M. Hof and W.T. Hermens).

Chapter 3 Implementation of Time-Resolved Fluorescence Correlation Spectroscopy (Paper II)

3.1 Hardware upgrade

The motivation of the presented hardware upgrade is to be able to simultaneously measure by a confocal microscope FCS data and a previously inaccessible fluorescence parameter – the excited state lifetime. The hardware upgrade required 3 main changes in the standard FCS setup[Paper II]:

1. replacement of cw lasers by high repetition pulsed ones (including suitable spectral filters and dichroic mirrors) - LDH-P-C-470 and LDH-P-635 with single PDL 800-B laser driver (PicoQuant GmbH, Berlin, Germany)
2. introduction of a fast TCSPC compatible single photon detector - SPCM-AQR-13-FC (Perkin-Elmer, Fremont, USA)

3. replacement of the online hardware correlator by a TCSPC card enabling to capture full dynamics of photon arrival in the so called Time-Tagged Time-Resolved (TTTR) mode - TimeHarp 200 (PicoQuant GmbH, Berlin, Germany)

Due to this upgrade every photon detection event can be stored with two relevant times – a macro time (100 ns resolution) from the start of the experiment and a micro time (40 ps resolution) from the laser pulse. This lossless data storing makes any arbitrary offline analysis possible.

3.2 Fast correlation algorithm enabling TR-FCS data treatment

The novelty of Time-Resolved Fluorescence Correlation Spectroscopy (TR-FCS) can be documented by the fact that no commercial software enabling TR-FCS exists. It means that we had to create our own routines for the data analysis. The first and the most important step was the development of fast correlation algorithm accepting photon weighting. The second step was a graphical interface providing users with a full data handling and TR-FCS analysis routines.

The difficulties connected with the software correlation are connected with huge amounts of data (up to 100 000 photons per second of experiment) and a large scale of correlation lag times (from 100 ns to seconds). The correlation algorithm development happened in several steps. Finally, the demands posed by the new ways of data analysis (photon weighting used in TR-FCS, experimental ACF weighting, markers from Z-scan, detectors routing in multi-focus setup) together with an inspiration taken from the recently appeared article[32] resulted in a variable algorithm fulfilling all the requirements and still keeping the calculation time much shorter than the measurement time.

3.3 Z-scan automation

The first Z-scan measurements were performed fully manually, because the Confocor 1 software does not enable the required automation of focus movement in z-direction alternated with FCS data acquisition. The full automation of Z-scan became possible after the upgrade of Confocor 1 by TimeHarp 200 board. We constructed a temperature controlled cuvette with a computer controlled linear

actuator for holding and moving the sample. Before and after the position change the linear actuator sends logical TTL signal that is registered by TimeHarp 200 board via its sub-D connector and inserted into TTTR data as a marker. The whole Z-scan experiment is thus saved into one TTTR data file with inserted markers, which enable to cut the measurement into pieces according to individual z-positions and thus perform Z-scan analysis.

3.4 Monte-Carlo simulations

As can be seen from the theory of TR-FCS, the mathematics behind it is rather complicated and difficult to imagine, especially the negative "intensity" of weighted photons entering the correlation calculations. To prove the concept of TR-FCS, to test the developed correlation algorithms and the graphical user interfaces Monte-Carlo simulations based on real experiments were performed.

The basic concept of the simulations was taken from Wohland[33], although some important changes needed for successful simulation of TR-FCS experiment were made. The parameters used for the computer simulations were as close as possible to the conditions used in the real membrane experiments.

For TR-FCS testing simulations, mixture of two non-interacting fluorescence species diffusing within a plane was used. They differed in the diffusion coefficient and in the excited state lifetime, their overall brightness was the same. The simulated data enable to calculate not only TR-FCS autocorrelation functions from the mixture, as in the real experiment, but also by knowing the exact photon origin, the routed autocorrelation functions of pure species.

The comparison of the calculated autocorrelation functions clearly shows that the filtered autocorrelation function is totally overlapping with the routed autocorrelation function, only the filtered one is noisier, which inherently comes from the statistical nature of its calculation. By this we proved that the concept of TR-FCS and our software for TR-FCS data analysis work.

3.5 Experimental testing

After *in silico* testing of TR-FCS, we performed two simple tests of the method by real experiments.

First we tested the ability of TR-FCS to separate the autocorrelation curves of dyes with similar diffusion coefficients, but with different lifetimes and thus simultaneously track their concentration in a mixture. In the second experiment we tested the utility of TR-FCS approach for obtaining the cross-correlation function in a single-channel measurement. Both experiments were successful and are documented in Paper II.

3.6 Applications (Paper XI, Paper XIV)

The basic applications of the presented hardware and software upgrade are simultaneous TCSPC and FCS experiments. Interpretation of FCS data alone is often tedious and requires additional experiments. Having simultaneously the lifetime information can help a lot in this situation. For example the excited state decay pattern gives direct information on the amount of non-correlated background light or reflected and Raman scattered light. It may identify impurities in solutions or reveal whether the studied fluorophore changes its lifetime during the FCS experiment.

In Paper XI we exploit the lifetime added value of the upgraded Confocor 1 setup together with the optimized sample holder for studying the nanoscale-confined diffusion of fluorescent probe molecules inside highly ordered alumina nanopores. The motivation was to restrict the 3D diffusion of studied molecules by the nanopores and thus reach the apparent 1D diffusion leading to longer observation times of the molecules. The ACF analysis really revealed the apparent 1D diffusion inside the pores with a dwell time in the confocal volume up to 19 times longer than in the case of the bulk 3D diffusion. Moreover the apparent concentration was lowered due to a smaller effective probe volume. Analyzing the fluorescence lifetime changes in the nanostructure, we found that no collisional quenching of fluorescence at the pore walls occurs. Our findings render one-dimensional fluorescence fluctuation spectroscopy (1DFCS) in ordered nanoporous alumina an efficient tool to study macromolecules in channel-type confinement and to evaluate the performance of these membranes in separation and sensing.

The simultaneous TCSPC and FCS experiments appeared to be very helpful in DNA condensation studies. The task of these studies is to find an efficient DNA condensing agent for application in non-viral gene therapy (NVGT). The condensing

agent is supposed to effectively pack large DNA molecules into so-called nanoparticles, deliver them into targeted cells and enable the cell transfection.

We concentrated on the first part of the NVGT, the efficient condensation of DNA molecules. We have shown that FCS is a suitable tool for testing condensers efficiency[34-36, Paper XII, Paper XIII]. The FCS data monitoring the condensation process are complicated and not still fully understood. The problems arise from the starting size of DNA molecule, which is larger than the focal volume, from its complicated conformation in the non-condensed form, and from the DNA fluorescent labeling usually performed by intercalating dyes, which are partially expelled from the double coil during the condensation process.

The combined TCSPC and FCS have substantially contributed to the data interpretation by the ability to distinguish between a dye release and a dye quenching[Paper XIV]. When the overall intensity goes down and the excited state lifetime remains constant, we observe dye release. As soon as the excited state lifetime is shortened proportionally to the intensity decrease, no dye release happens; only the environment of the intercalated dye in DNA strand is changing, leading to a decrease of the quantum yield of the dye.

Among advanced options of the presented upgrade belong applications of TR-FCS. Two of them, using multiple labeling, were mentioned in the previous section. Another simple, but useful, application of TR-FCS is a removal of detector afterpulsing from ACF[37]. However, the main power of the method lies in systems, where we study kinetics connected with a lifetime change of fluorophore. For example one can study rate constants of the opening and closing of the double-stranded terminal segments of cTAR[38] by introducing a fluorescence label to one end of the cTAR and a fluorescence quencher to the second end (so called beacon). As the cTAR is opening and closing, the lifetime of the fluorophore is changing and the TR-FCS should be able to study its kinetics. This work is currently under progress.

Another already worked out application of TR-FCS is described in the next chapter.

Chapter 4 TR-FCS combined with lifetime tuning (Paper III)

Since the fluorescence lifetime of a chromophore is sensitive to changes in its particular microenvironment or to intentional addition of quenching channels, TR-FCS offers the unique advantage to simultaneously characterize different processes using single dye labeling. If the same dye is located in two well defined environments, the environments cannot be physically separated to a distance larger than the optical resolution and in each environment the dye has a different excited state lifetime, TR-FCS enables to calculate the autocorrelation function for each environment from a single experiment. We exploited this important feature of TR-FCS by measurements on SPBs[(Paper III)].

It is well known that the presence of an optical interface (in case of SPBs the solid support) modifies a lifetime of a fluorophore close to it. In our case this means that a labeled lipid embedded in SPBs has a different lifetime than the same lipid embedded in vesicles freely diffusing in solution. To make a practical use of this feature we first investigated how the lifetime of the labeled lipid in SPBs depends on the support and compared the relative lifetimes with theoretical calculations. The optical constants of the used surfaces needed for calculations were measured in air by variable angle spectroscopic ellipsometry, the theoretical calculations were provided by J. Enderlein (Jülich, Germany). A very good agreement between the theory and the experiment was found, giving also the average inclination angle of the dye dipole respective to the surface plane. It was confirmed that the nonadsorbing media have only a moderate effect on the lifetime, whereas the light absorbing media (metals and semiconductors having a complex refractive index) vary the lifetime of a dye in their proximity a lot.

In the second step we studied the distance dependence of the quenching phenomena for oxidized silicon and glass covered with indium-tin oxide (ITO) layer. Using the Langmuir-Blodgett stacking technique odd numbers of DPPA monolayers in the gel phase were used as variable spacers between the support and the last labeled fluid monolayer. Again the experiments were compared with calculations

giving agreement on a steep distance dependence of the quenching efficiency. This means that only a dye very close to the surface, in our case embedded in SPBs, is substantially quenched, while the lifetime of the dye in vesicles in solution remains almost unchanged.

In the last step we showed that after preparing SPBs from labeled vesicles on ITO and oxidized silicon, TR-FCS enables to characterize simultaneously 2-dimensional lipid diffusion in SPBs and 3-dimensional vesicle diffusion in a single experiment. Diffusion coefficients obtained by this new approach were compared to diffusion coefficients obtained by standard FCS on isolated SPBs or vesicles, respectively.

Lateral diffusion of weakly bound proteins, like e.g. the blood coagulation protein prothrombin, is very difficult to characterize. Since the free and bound proteins are in equilibrium flushing away the free protein does lead to desorption of the protein, and thus, the only-bound-protein system cannot be reached. It means that under any experimental condition the fluorescence signal from the free protein always interferes with the signal coming from the bound protein. However, the lifetime of a dye bound to the membrane-bound protein might be controlled by the here demonstrated approach. TR-FCS might then in analogy to the here demonstrated vesicle experiments allow to simultaneously characterize 2-dimensional protein diffusion on SPBs and 3-dimensional protein diffusion in bulk. Moreover, cross-correlation analysis might give direct information on protein binding rates.