Part III

Summary and Conclusions

Chapter 8

General summary, conclusions and outlook

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8.1 Abstract

In the previous chapters, the development and applications are described of important fluorescence-based techniques to measure diffusion of biomolecules. New insights were obtained on the diffusion of molecules inside cells and cell membranes, and through protein pores. This section briefly summarizes the most important findings with emphasis on the techniques used. In addition, a number of recent developments in fluorescence spectroscopy are discussed and their potential to study diffusion in biological systems.

8.2 Dual-color fluorescence-burst analysis

Part I of this thesis presents **d**ual-**c**olor **f**luorescence-**b**urst **a**nalysis (DCBFA), a new technique that can be used to determine the functional and structural properties of membrane pores. DCFBA enables to quantitatively determine the internal concentration in liposomes of fluorescently-labeled size-marker molecules. DCFBA allows to study leakage from liposomes and this makes it an excellent technique for studying membrane pore-forming proteins. Chapter 2 presents the DCFBA method with emphasize on the technical details.

In chapter 3 [17], DCFBA was successfully used to determine the effective pore-size of the mechanosensitive channel of large conductance MscL from *Escherichia coli*. Compounds smaller than 6.5 kDa were able to pass through MscL, whereas larger macromolecules were not. Using the single liposome resolution of DCFBA, we were able to determine that the reconstitution efficiency of MscL was less than 10%. Thus, about 90% of the channel must have been lost during the reconstitution and / or not functionally incorporated into the liposomes. In addition to reconstitution efficiencies, the single liposome resolution of DCFBA can be used for many other applications. One interesting idea is to apply DCFBA to characterize the expression of fluorescent proteins, using whole bacterial cells, similar to the experiments with *Lactococcus lactis* from chapter 4.

In chapter 4 [19], DCFBA was employed to study the pore-forming mechanism of the antimicrobial peptide melittin from bee venom. It was found that the mechanism of pore-formation by melittin depends on the lipid composition. An interesting idea is to perform DCFBA experiments with fluorescently labeled antimicrobial peptides, because then the fluorescence intensity will be proportional to the number of antimicrobial peptides. This would provide information on the stoichiometry of the pore and how many peptides are needed for pore-formation. Also, in principle, the kinetics of the pore-formation can be followed by speeding up the data acquisition by laser-scanning or sample stirring (chapter 2), and this can be used to determine the life-time of the pores.

In conclusion, DCFBA enables to measure leakage of fluorophores from liposomes and it can be used as an universal method to study translocation of fluorophore-labeled (macro-) molecules through membrane pores, expressed in cells or reconstituted in liposomes. Measurements can be done with a small amount of material (1 – 10 µg liposomes) and in a few seconds time span, provided the acquisition is sped up using probe-scanning or sample stirring. Thus, DCFBA allows for medium-throughput screening of lipid compositions and size-marker molecules. More importantly, information on the population level of liposomes is obtained and not just the average extent of leakage. Furthermore, one can distinguish between membrane fusion / aggregation, membrane lysis and pore formation in a single experiment. Binding to membrane proteins can also be measured with DCFBA (explained in chapter 2), and this can be applied to obtain information on the specificity, stoichiometry and cooperativity of these binding reactions. These merits make DCFBA an important technique to study membrane pores. It can be used not only to obtain structural and functional information on integral membrane channels (chapter 3) and antimicrobial peptides (chapter 4), but also on other membrane active com-

pounds, such as detergents, drugs (*e.g.* the anti-malarial drug chloroquine [292]), cell permeating peptides (*e.g.* the Tat-peptide from the HIV-virus [293]), and membrane disrupting peptides (*e.g.* amyloid-beta that causes Alzheimer's disease [294]).

8.3 Fluorescence correlation spectroscopy

Part II of this thesis presents diffusion measurements using **f**luorescence **c**orrelation **s**pectroscopy (FCS) and **f**luorescence **r**ecovery **a**fter **p**hoto-bleaching (FRAP).

In chapter 5 [21], FCS was used to study the influence of sugar on the lateral mobility of phospholipids. It was found that sucrose decreased the lateral mobility of phospholipids more than other sugars did, including trehalose. Molecular dynamics simulations showed that this was due to more hydrogen bonds between sucrose and the lipid head-groups compared to trehalose. Thus, the precise molecular interactions between the sugar and the lipids have a large influence on the mobility of the lipids. This is of particular biological relevance, since living cells contain a dense and complex mixture of molecules [215]. The many electrostatic and steric interactions will have a major impact on the diffusion of both soluble and membrane associated molecules. Indeed, typical diffusion constants of membrane proteins in physiological membranes are at least 10-fold lower (~ 0.3 μm^2 s^{-1} in fibroblasts [295]) than in synthetic membranes (~ 3 μm^2 s^{-1} [9]), which is due to membrane protein crowding [295].

The slow diffusion of macromolecules *in vivo* can result in artifacts in the FCS measurements, because of photo-bleaching by the focussed laser. In addition, the presence of fluorescent aggregates, macroscopic motion (*e.g.* sample movement, membrane undulations) and the nearby presence of macroscopic stuctures, such as organelles, pose other difficulties to FCS. FCS measurements on the mobility of membrane (associated) molecules is especially prone to artifacts, because of their slow (10 – 100-fold lower) mobility compared to soluble proteins. Recently, a number of new techniques have been developed to overcome these problems. For instance, by scanning the focal volume through the sample, either parallel [35] or axial [296, 297] to the membrane, one reduces the influence of photo-bleaching and increases the statistical accuracy. Moreover, it makes measurements in undulating membranes possible. Also, scanning can lead to higher accuracy of the FCS data, since the scanning speed and frequency can be used for the calibration of the detection volume [298].

Because of the high crowding and spatial heterogeneities inside the cell, the mobility of macromolecules can vary in place and time. This results in a nonlinear

relationship of the mean square displacement as a function of time, and this is called anomalous diffusion [299]. The development of fluorescence microscopy techniques to measure below the diffraction-limited resolution, using multiple photon excitation or active depopulation from the excited state (**st**imulated **e**mission **d**epletion, STED, reviewed in [300, 301]), might be useful to study anomalous diffusion. These techniques have lateral resolutions as low as 25 nm [302], and might be combined with FCS to test the extent of the anomalities at very short spatial distances that cannot be studied with conventional confocal microscopy ($\sim$ 200 nm lateral resolution). Importantly, the first commercial version of a STED microscope (from Leica, Wetzlar, Germany) has recently hit the market.

8.4 Fluorescence recovery after photo-bleaching

The effect of high crowding of macromolecules on their mobility is presented in chapter 6 [22], where a pulsed version of FRAP (pulsed-FRAP) is described to determine the mobility of GFP in *E. coli*. In *E. coli* the crowding of macromolecules is very high, and the concentration of proteins, DNA and RNA account for $\sim$ 25 to 30% of the cell volume [214]. The diffusion in bacteria cannot be accessed with conventional FRAP (see section 1.4), because of their small size ($\sim$ 2 μm for *E. coli*), which is close to the diffraction limit of optical microscopy. Because the cell size and geometry of the bacterial cells are taken into account, pulsed-FRAP allows to measure diffusion in bacteria. In addition, since only a relatively small fraction of the fluorophore is photo-bleached, pulsed-FRAP allows multiple measurements in the same cell. It also enables diffusion measurements with relatively photo-unstable fluorophores.

In chapter 6, we report that upon an osmotic upshock from 0.15 to 0.6 Osm, the apparent intracellular diffusion of mobile GFP in *E. coli* cells decreases from 3.2 to 0.4 $\mu m^2\ s^{-1}$. Exposing *E. coli* cells to higher osmolalities ($>$ 0.6 Osm) led to compartmentalization of the GFP into discrete pools, from where the GFP could not escape. Pulsed-FRAP can be applied for measurements of diffusion of other biomacromolecules, differing in size and surface properties, and small fluorophores, in small bacterial cells or eukaryotic organelles. In addition, the fitting model (Eqn. 6.3 – 6.6) might be modified to allow for measurement of lateral mobilities of membrane associated molecules. The main limitation of pulsed-FRAP is that only fluorescence from the position of the focussed laser is taken into account. A major improvement would be to employ a whole-cell approach and combine FRAP with confocal imaging to use information on the fluorophore content and distribution from the whole cell,

as was used in [218, 219, 220] and in chapter 7.

Selective-FRAP is a fluorescence-based technique that allows to measure fluorophore mobility between compartments, *e.g.* organelles [16]. It is based on the (partial) photo-bleaching of fluorophores in a compartment and subsequent recording of a time-series of images to monitor the net influx of unbleached molecules. In chapter 7, we describe a quantitative version of selective-FRAP to measure the mobility of GFP-tagged reporter constructs between the nucleus and the cytoplasm in life yeast cells. Using this method, a new mechanism was found to target protein synthesis to the emerging daughter cell during cell division. Similar to pulsed-FRAP, the fitting model for selective-FRAP (Eqn. 7.2) can in principle be modified for studying the transport of membrane proteins between subcellular compartments.

A major problem of FRAP is that photo-bleaching takes place not only during the photo-bleaching step, but also during the measurement of the recovery of the fluorescence. Thus, in the selective-FRAP experiments of chapter 7, a (small) fraction of GFP was photo-bleached during the confocal imaging of the recovery time-series. An interesting idea is to use this photo-bleaching during confocal imaging to measure diffusion in the following way: A confocal image is acquired pixel by pixel, by translating the focal volume in the x and y-directions (perpendicular to the optical axes), with dwell time t_d (Fig. 8.1a). Acquiring a confocal image takes time (t_d times the number of pixels) and diffusion takes place during this imaging. Because of diffusion, the photo-bleached fraction is larger at the end of the imaging compared to the beginning of the imaging. Thus, the fluorescence intensity at the end of the confocal image will be lower than that at the beginning, and this asymmetry becomes larger if t_d is increased. Indeed, dividing two images of an *E. coli* cell expressing GFP with two different acquisition times t_d resulted in (slight) asymmetry (Fig. 8.1b – c). In principle, the diffusion coefficient of GFP inside the bacteria can be calculated by comparing confocal images with different dwell-times t_d, and using a similar computational approach for the data analysis as described in chapter 6 [22]. Unfortunately, one would like to compare very slow imaging ($t_\mathrm{d} \approx 1$ – 10 ms) with very fast imaging ($t_\mathrm{d} \approx 1$ – 100 µs, as in [65]) and this is not feasible with the current microscopy setup in the group. In general, it is often overlooked that confocal images also contain temporal information [64, 65].

8.5 Concluding remarks

In the first part of this thesis, DCFBA was developed to study diffusion through membrane pores. In the second part, FCS and FRAP-based techniques were used

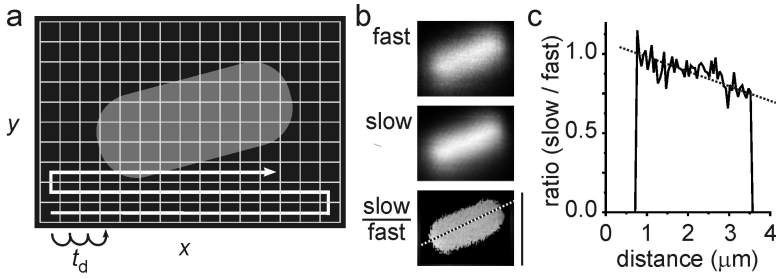

Figure 8.1: Confocal imaging to determine mobility of fluorophores *in vivo*. (a) A confocal image is recorded pixel by pixel, by scanning the focal volume in lateral directions x and y (white arrow, perpendicular to the optical axis) with dwell-time t_d. Therefore, a confocal image not only contains spatial but also temporal information, which can be used to calculate diffusion coefficients by combining it with FCS [64, 65] of FRAP (panel b). (b) Proof of principle of confocal imaging combined with FRAP. Two images of an *E. coli* cell were recorded with different dwell times t_d: 1 ms (fast) and 2 ms (slow). Dividing the intensities of each pixel of the two images resulted in asymmetric ratios, with a gradual decrease from the lower left to the upper right corner ($\frac{\text{slow}}{\text{fast}}$). This asymmetry is due to diffusion of the GFP and in principle the diffusion coefficient can be calculated from these ratios. Scale bar, 2 μm. (c) The ratio of the intensities as indicated in panel b. The dotted line indicates the gradual decrease of the ratio.

to measure diffusion in cells and cell membranes. The work presented in this thesis illustrates that fluorescence spectroscopy is an extremely powerful and versatile tool to probe molecular diffusion in biology, and various approaches can be used to meet the experimental conditions, such as the concentration of fluorophores and the spatial goniometry of the system. In the near future, the recent breaking of the diffraction barrier and the possibilty to image at very high resolution (reviewed in [300, 301]) might make it possible to determine mobilities at very small spatial scales. In addition, an increasing number of fluorophores is becoming available with characteristics that make them more suitable for diffusion measurements, *i.e.*, fluorophores with an increased quantum yield, photo-stability, solubility and / or membrane permeability (*e.g.* [303]). Important is the recent development of photostable, monomeric fluorescent proteins with various spectral properties [304] and of small fluorophores which can be used to selectively label short protein tags, such as the tetracysteine tag [281, 282, 283] (used in chapter 7) and the SNAP tag [305]. Lastly, numerous improvements are being made in the electronics, for instance faster computers and more sensitive detectors. Interesting is the ongoing miniaturization of the electronics, that enabled to image fluorescence in the hippocampus of freely moving mouse [306].

In conclusion, fluorescence spectroscopy is a rapidly developing field and becoming increasingly important in biology. Indeed, the number of scientific papers with 'fluorescence' as a topic was steadily growing over the last 20 years (Fig. 8.2, ■). The scientific output for other fields of research was also growing during this time, but this growth is clearly correlated to the stock indexes (Fig. 8.2, Dow Jones index, solid line). For instance, the number of papers with as topic 'electron microscopy' (Fig. 8.2, □) or 'crystal structure' (Fig. 8.2, •) was clearly affected by the early 2000s bursting of the technology bubble (`dot-com` slowdown, see the decrease of the Dow Jones index). This correlation between the number of papers and the stock indexes is probably due to the level of research funding: an economic recession leads to a decrease in research funding which affects the scientific output. Importantly, the number of papers for 'fluorescence' was not affected by the `dot-com` slowdown, and showed steady growth between 2000 and 2005. Thus, the field of fluorescence was maintaining a steady growth even in times of economic slowdown, and this is probably due to the ongoing technical breakthroughs and new discoveries in this field. The fact that fluorescence research is relatively insensitive to the state of the economy illustrates its increasing importance to science.

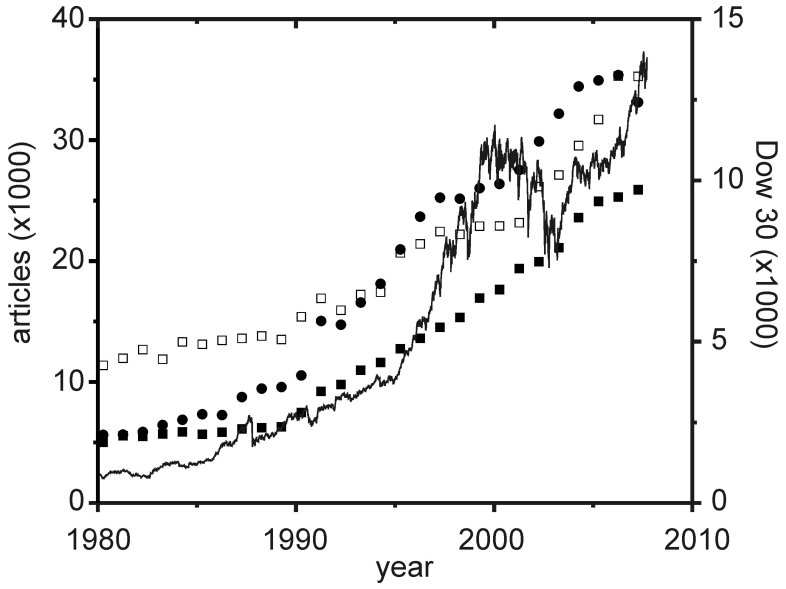

Figure 8.2: Scientific output correlates with the state of the economy. Left axis: the number of articles in the *ISI web of Knowledge* database (`http://apps.isiknowledge.com/`) with 'fluorescence' (■), 'electron microscopy' (□) or 'crystal structure' (●) as a topic. Right axis: daily averages of the Dow Jones industrial average index. *ISI web of Knowledge* contains a very broad collection of scientific literature, including biological, chemical and physical papers.

8.6 Acknowledgements

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