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Membrane heterogeneity : from lipid domains to curvature effects

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Membrane heterogeneity

From lipid domains to curvature effects

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“There is never any end. There are always new sounds to imagine; new feelings to get at. And always, there is the need to keep purifying these feelings and sounds so that we can really see what we’ve discovered in its pure state. So that we can see more and more clearly what we are. In that way, we can give to those who listen the essence, the best of what we are. But to do that at each stage, we have to keep on cleaning the mirror.”

John Coltrane (1926-1967), Jazz saxophonist

Für meine Familie

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CHAPTER 1

INTRODUCTION¹

The length scales studied in experiments today comprise many orders of magnitude: from subatomic structure unraveled by large particle accelerators to galaxies many lightyears away imaged by space born telescopes. Despite the technical sophistication, which makes this wide range accessible, there are still systems that defy the resolution of their organization on a micrometer to nanometer scale. One of them is the cell membrane. The sheer complexity of this nanometer-thin fluid material has been challenging biologists and physicist for many years. One of the first models that captured essential features of the cell membrane is the homogeneous fluid mosaic model (1). This model was refined by including heterogeneity (Fig. 1.1), where either the lipid composition or the proteins were given the leading role. Both concepts are in the process of being reconciled in the light of new experiments on lipid-protein interactions. Those interactions range from specific chemical to unspecific and purely physical. The latter comprise membrane curvature mediated interactions which have recently been shown to influence a large number of biological processes. In parallel to the conception of refined models, new experimental techniques to determine membrane microstructure were developed. Single molecule fluorescence has emerged as one of the leading technologies since it delivers the required spatial resolution and can be employed in living cells. In a

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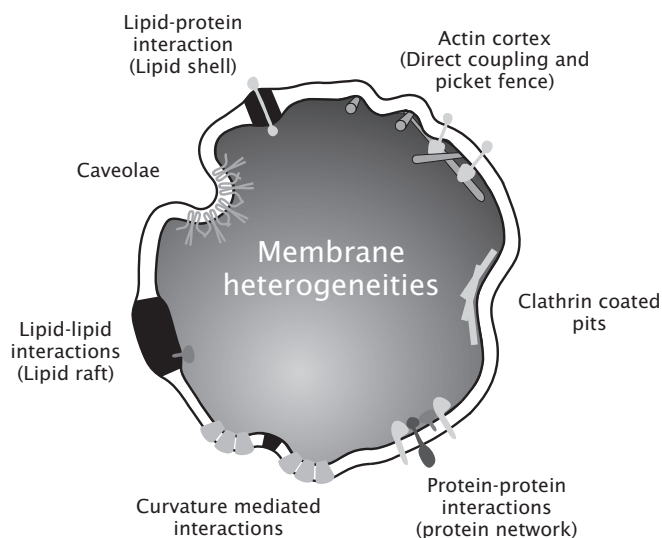


Figure 1.1
Membrane heterogeneities

complementary approach artificial model systems are used to study specific biophysical aspects of membranes in isolation and in a controllable way. Nowadays, artificial membranes have outgrown their initial status as simplistic mock cells: a rich spectrum of different phases and phase transitions and the unique possibility to study membrane material properties make them an exciting subject of research in their own right (2, 3). In this thesis we show how phase separated artificial membranes can be used to gain fundamental insight into lipid composition based heterogeneity (Chap. 2) and membrane mediated interactions (Chap. 3). We demonstrate that those interactions can lead to lipid domain sorting (Chap. 4). Experiments with artificial membranes are complemented with live cell studies. We develop a robust analysis method for single molecule position (Chap. 5) and use it to study the role of heterogeneity in cell signaling (Chap. 6). Finally, we show how protein cluster formation can be measured by counting single molecules in live cells (Chap. 7).

1.1 Membrane heterogeneity

The plasma membrane is a complex, self assembled composite material fulfilling a host of different functions. Historically, the membrane was mainly considered a semi-permeable barrier necessary for maintaining biochemical conditions that are different from the environment. Homeostasis and metabolism require highly selective permeability for certain molecules, which is provided by ion channels and active transporters. While membrane asymmetry - a difference in composition between the two leaflets of the lipid bilayer - might influence membrane permeability, the barrier function of the membrane does not imply any lateral structure. Correspondingly, early models of the membrane sketched it as a homogeneous, liquid mosaic (1). As more and more elaborate functions of the cell membrane were identified, this image of the membrane structure had to be refined (4). Being much more than a simple barrier, the membrane serves as a two-dimensional reaction platform for a plethora of biochemical reactions. For these reactions to take place quickly and efficiently, the membrane was suggested to be compartmentalized (5–7). In this way the composition of local environments could be optimized for the functioning of certain membrane proteins. (8, 9). For example, some G protein coupled receptors translocate after ligand binding to specific microdomains, where they interact with their G protein (10, 11) (Chap. 6 of this thesis). Confinement to small domains diminishes the time for a receptor and co-factors to meet and therefore speeds up signaling (12). Microdomains also serve as platforms for receptor internalization and therefore desensitization (13). Furthermore, they are speculated to serve as entry ports for viruses (14) and to play a vital role in immunology (15). While many biological functions have been shown to depend on local environments with specific compositions, the physical nature and the driving forces for their formation are still discussed and many different kinds of microdomains have been identified, see Fig. 1.1.

The "lipid raft" model champions lipid composition as the major driving force for heterogeneity (8, 9). A lipid raft was thought to be a membrane domain which is enriched in sphingolipids and cholesterol (liquid ordered phase), see Fig. 1.2. Artificial membranes made from (unsaturated) phospholipids, (fully saturated) sphingolipids and cholesterol spontaneously phase separate into a (liquid ordered) "raft" phase and a liquid disordered phase. These phases, which differ in their lipid tail organization, have the ability to differentially segregate proteins *in vitro* (16, 17).

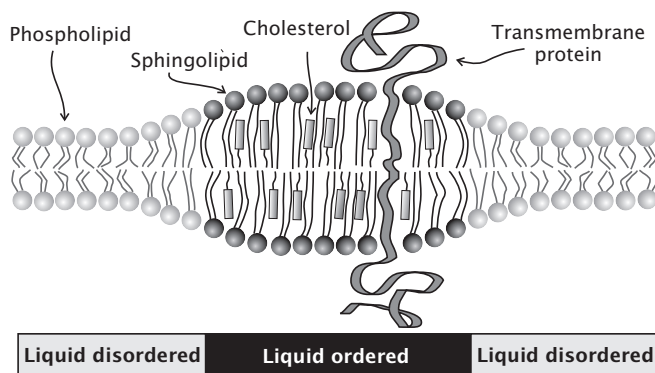


Figure 1.2

Liquid ordered and liquid disordered phase differ in composition, lipid tail order and thickness

Biochemical assays on cells, based on detergent resistance and cholesterol depletion, seemed to suggest that certain proteins can be found in such domains *in vivo* too. The significance of those experiments is, however, questionable (18): detergent and cholesterol depletion are suspected to induce the formation of domains. Additionally, lipid rafts were found to be at most a few tens of *nm* in diameter, which means they could transport only a few proteins (19, 20) (Chap. 2 of this thesis). Furthermore, the binary picture of the cell membrane divided in raft and non-raft regions is oversimplified: Not only that the membrane contains significantly more than 3 different types of lipids which could result in several liquid phases all with different properties. Even with only 3 components, membranes that are asymmetric - the compositions of the two leaflets are different - exhibit 3 distinguishable phases (21). Also the idea that rafts are stable entities was challenged (19). Recent experiments (22, 23) suggest that the membrane of a live cell at physiological temperatures is close to a miscibility critical point which implies strong composition fluctuations. The question is then, whether these fluctuations last for a long enough time to cause significant protein segregation. Composition fluctuations could be coupled to and stabilized by membrane shape fluctuations (24). Remarkably, heterogeneous model systems without any visible phase separation (i.e. above the miscibility critical temperature) were reported to show inhomogeneity on a length scale of tens of nanometers (25). The experiments discussed above are a few examples for recent work that has

led to doubts about the original "lipid raft" concept and to a more nuanced definition of "membrane rafts": "Membrane rafts are small (10 - 200nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions." (26).

If lipid composition is not driving membrane heterogeneity, could proteins be responsible for the formation of domains? The fact that the membrane is highly crowded (27) implies that protein-protein interaction play a dominant role. Indeed it was shown that proteins can form microdomains without involvement of lipid rafts (28). Historically, clathrin coated pits - membrane dimples caused by a cage of clathrin molecules - and caveolae - membrane invaginations formed by crosslinked caveolin molecules - were the first membrane micro domains to be identified. Moreover, the influence of proteins is not restricted to membrane proteins, the underlying cytoskeleton is believed to interact with the membrane, as well (29–34). A passive, flexible cytoskeletal network can cause membrane domains by exerting a force and deforming the membrane locally (33). In this model diffusion is influenced by steric repulsion between the network filaments and molecules in the membrane. A dynamic actin network has been shown to actively drive phase separation by the coupling of polymerization to membrane proteins that have spontaneous curvature (31, 32). In a different model, the "picket fence", the cytoskeleton affects the membrane indirectly via anchored transmembrane proteins. Those "pickets" are supposed to transiently confine proteins and lipids to nanometer sized domains (35). This idea has been challenged, by recent state of the art single-molecule fluorescence experiments (36), which do not find any confinement on length scales reported previously.

It seems that membrane heterogeneity cannot be ascribed to either lipids or proteins alone. Only if interactions between lipids and proteins (and other membrane constituents) are considered, the picture will be complete. Examples for these interactions are numerous. Proteins might assemble a shell of lipids around them (37, 38) whose size is comparable to possible "rafts" caused by lipid-lipid interactions (20) (Chap. 2 of this thesis). Crosslinking of GM1, a ganglioside and supposed lipid raft component, can induce phase separation in model membranes (39). The same happens if an actin network - as a model of the cortical cytoskeleton - is polymerized on the membrane (40). Proteins and peptides also

influence the line tension between coexisting lipid phases by binding to the domain boundary(41). For all these mechanisms for protein-lipid interaction to work the membrane could be a flat sheet. In fact, the plasma membrane and, especially, the membranes of inner organelles are curved with radii of curvatures from a few nm to μm (42–44). Curvature has been shown to influence processes like endocytosis and protein coat assembly (45–48) and phase separation of lipids (49). Since a lipid bilayer is elastic and resists bending (2) the question is, how membrane curvature is realized. As with the creation of heterogeneity, both lipids and proteins are involved (50). Membrane curvature affects lipid packing (51) and lateral lipid distribution (52). Also proteins interact via the curvature of the membrane (53–55), which can lead to sorting and thereby promotes heterogeneity (52, 56) (Chap. 4 of this thesis). Effects related to membrane curvature sensibly depend on material properties of the membrane, like bending rigidity and spontaneous curvature, which are functions of the lipid composition (22, 57) and depend on the properties of the extracellular matrix (58). Curvature is therefore another intermediary for the interplay of proteins and lipids: proteins modulate membrane curvature, lipids redistribute to regions with high or low curvature, changing the local composition, which influences curvature mediated effects and the curvature itself.

As the examples given above illustrate, there are many mechanisms by which membrane heterogeneity can be created. In a living cell all these processes take place at the same time and influence each other. Model membranes, however, allow one to study single processes separately in a controlled environment.

1.2 Artificial membranes

The influence of lipid composition on physical and chemical properties of the membrane can best be studied in a model system which allows complete control over the membrane's composition. To that end, giant unilamellar vesicles (GUVs) have proven to be one of the most versatile model membrane system. These closed, spherical single lipid bilayers of 10-100 μm diameter can be produced from a broad range of lipid compositions in physiologically relevant buffer conditions (59). They are free-standing and can be produced in sizes that are comparable to cells. While macroscopic (micrometer sized) lipid domains are absent in living cells, they can be

readily reconstituted and studied in GUVs (60). Alternatively, membrane domains are studied in supported lipid bilayers. However, it was found that interaction with the support influences the thermodynamic behavior of domains (61). In contrast to domains in GUVs, which are mobile and frequently fuse, the domains reconstituted in supported bilayers are practically static. The observed long term stability of domains corresponds therefore to a non-equilibrium situation. For these reasons we will restrict our discussion in the following to free-standing GUVs.

1.2.1 Formation and observation

Electroformation of GUVs Most commonly, GUVs are made by electroformation (62), a technique that provides a high yield of unilamellar vesicles of controllable lipid composition and size. Briefly, lipids are dissolved in an organic solvent and dried on the plates of a capacitor. The space between the plates is filled with an aqueous buffer and an oscillating electric field is applied. On the time scale of hours the electric field causes vesicles to swell from the lipid film and continue to grow by fusion. With electroformation it is possible to produce vesicles that consist of a mixture of phospholipids, cholesterol and sphingolipids, mimicking the composition of a cell membrane (60). In contrast to the cell membrane, however, there is no difference in composition between the leaflets in an electroformed vesicle. Also, in cells lipids are often attached to a carbohydrate (glycolipids), which is not the case in artificial membranes.

Below a certain critical temperature heterogeneous membranes spontaneously phase separate into two fluid phases, which requires the electroformation to be performed at a temperature above this critical temperature. Electroformation in its original form, pioneered by Angelova et al. (62) was not compatible with buffers of physiological salt conditions. This drawback can be overcome by the use of charged lipids (63), the exchange of the buffer in a flow chamber (64), or by using electric fields oscillating with much higher frequencies than in the original method (65). The approach devised by Montes et al. (65) permits the formation of GUVs from native membranes while preserving the compositional asymmetry between the leaflets, which is an important characteristic of biological membranes.

Microscopy on GUVs Due to their big size, GUVs are ideal for observation with standard microscopy techniques like phase contrast (59). From the vesicles' average shape (66) and the magnitude of thermal shape

fluctuations (67) important membrane material parameters, like bending rigidity and surface tension, can be obtained. The change of these material parameters can be followed through phase transitions (68) and their dependence on membrane composition can be studied (57). The use of artificial membranes for elucidating lipid driven heterogeneity was spurred by the work of Dietrich et al. (60, 69). They devised a method to directly visualize different liquid phases in phase separated GUVs, exploiting the differential partitioning of fluorescent probes. This method allowed the study of phase diagrams of phase separated membranes (3), see Fig. 1.3, and membrane material properties (20, 70, 71) (Chap. 2 of this thesis). The lateral lipid distribution on a molecular scale, however, is still inaccessible to current experimental techniques and can only be studied *in silico* (72).

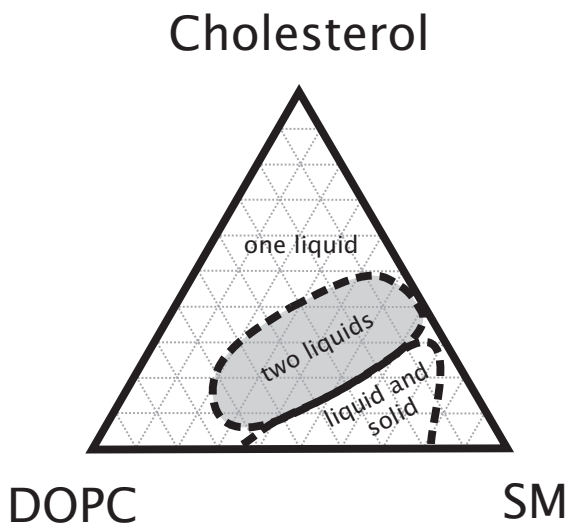


Figure 1.3

Phase diagram of a tricomponent vesicle consisting of phospholipids (DOPC), sphingolipids (sphingomyelin, SM) and cholesterol. Figure adopted from (73)

1.2.2 Selected results

Phase separation and material parameters Below a certain critical temperature, which depends on composition, heterogeneous vesicles phase separate (60). Such phase separated systems are most relevant to biology if the phases are both liquid. Tricomponent vesicles made from phospholipids, sphingolipids and cholesterol indeed exhibit a region of coexistence of two liquid phases, see Fig. 1.3, even at physiologically relevant tem-

peratures (73). After quenching the system from the homogeneous phase to the coexistence regime, the phase separation evolves by nucleation and domain coalescence or spinodal decomposition and coarsening (74).

While the two phases, named liquid ordered (L_o) and liquid disordered (L_d), are both characterized by a high lateral mobility of the lipids (75), they differ in the organization of the lipid tails, see Fig. 1.2. In the L_o phase, which is enriched in sphingolipids and cholesterol, the cholesterol intercalates between the sphingolipids and causes long range correlation between the lipid tails, hence this phase is called ordered. In the L_d phase which contains predominantly phospholipids, neighboring lipid tails interact only weakly and, due to the kink in the unsaturated acyl chains, there are more tail configurations possible. Because of the lack of orientational correlation between the tails this phase is called disordered.

The existence of two coexisting phases implies an energy connected to the interface between them. The interfacial energy per unit length is called line tension τ . Since the total energy of the interface is proportional to the length of the interface, the energy of a vesicle is decreased by coalescence of domains. The ground state should therefore be a completely phase separated vesicle, as shown in Fig. 1.4. Since the two phases differ in thickness (76), lipids at the interface have to bend or stretch, respectively, in order to avoid hydrophobic mismatch (77, 78), see Fig. 1.2. This mechanical energy contributes significantly to the line tension.

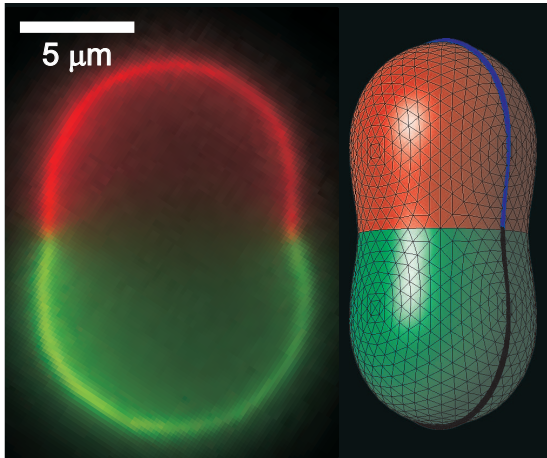


Figure 1.4

Left: Fluorescence microscopy image of a fully phase separated vesicle (equatorial section). The line tension manifests itself in the peanut shape (L_o phase shown in red, L_d phase shown in green); Right: Vesicle shape determined numerically from Eq. 1.1 with fitted analytical model for the contour (blue and black).

From a continuum point of view, the two phases can be characterized

by bulk material properties: surface tension σ , bending rigidity κ and Gaussian bending rigidity κ_G . For asymmetric membranes, the spontaneous curvature is another parameter to be considered; it gives the preferential curvature due to different composition of the two leaflets. The surface tension σ gives the energy needed to increase the membrane area with a fixed number of lipids. The concentration of free lipids in an aqueous buffer is negligibly low such that the number of lipids can be considered constant. In a relaxed vesicles (low-tension regime), the surface tension varies only weakly with vesicle surface area. This is due to the existence of thermal membrane fluctuations which are stretched out at increased tensions (79). In a tense vesicle the surface tension increases linearly with area, corresponding to an increased surface area per lipid. The shape of a vesicle also depends on the pressure difference between inside and outside, the Laplace pressure p .

While surface tension σ and pressure p are properties of individual vesicles, κ and κ_G only depend on membrane composition. Since the composition of the two phases changes with temperature, κ and κ_G effectively depend on temperature. The line tension strongly depends on the composition of the two phases -it vanishes at the critical point - and therefore on temperature as well (22), and it might also be influenced by lateral (surface) tension (80). The influence of all the material parameters are considered in the Canham-Helfrich energy (81, 82):

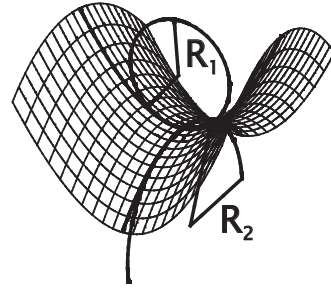


Figure 1.5
Principal radii

$$\mathcal{E} = \sum_{i=1,2} \int_{S_i} \left(2\kappa_i H^2 + \kappa_G^{(i)} K + \sigma_i \right) dA - pV + \tau \oint_{\partial S} dl, \quad (1.1)$$

The subscript i refers to the two phases, $H = 1/R_1 + 1/R_2$ the mean curvature and $G = 1/(R_1 R_2)$ the Gaussian curvature. R_1 and R_2 are the principle radii, see Fig. 1.5. Integration is over the surface of the phases S_i or the interface ∂S respectively. The shape of a vesicle can be found by minimization of this energy. As obvious from Fig. 1.4, the line tension is of prominent importance, leading to full phase separation and the characteristic 'peanut' shape.

Measurement of the thermal membrane fluctuations of the two phases allows the determination of the bending rigidities κ_i and surface tensions σ_i . For an infinite, two-dimensional, flat membrane in thermal equilibrium, the spectrum of out-of-plane fluctuations is

$$\langle |u_{\vec{q}}|^2 \rangle = \frac{1}{L^2} \frac{k_B T}{\sigma q^2 + \kappa q^4} \quad (1.2)$$

which follows from the Canham-Helfrich energy (82) and the equipartition theorem assuming periodic boundary conditions with period L . $u_{\vec{q}}$ is the Fourier component with wave vector $\vec{q}$. With the microscope we always observe a projection of the membrane which results in a one-dimensional contour, see Fig. 1.6, and a one-dimensional fluctuation spectrum. In phase separated vesicles we can use only the area around the vesicle poles for fluctuation analysis. The reason is that there, far away from the interface, the vesicle is very nearly spherical and the fluctuations can be measured relative to the mean radius (20) (Chap. 2 of this thesis). Using only a contour section of length a leads to a modification of the theoretically expected spectrum (83). Finally, the finite integration time for observation has to be taken into account, which influences the measured magnitude of long wavelength fluctuations, since those have a long correlation time (67). By consideration of all these effects the power spectral density of the shape fluctuations reads

$$\begin{aligned} \overline{\langle |u_k|^2 \rangle} &= \underbrace{\sum_{q_x} \left(\frac{\sin((k - q_x) \frac{a}{2})}{(k - q_x) \frac{a}{2}} \right)^2}_{\text{finite contour section}} \times \\ &\underbrace{\frac{L}{2\pi} \int_{-\infty}^{\infty} dq_y}_{\text{projection}} \underbrace{\frac{k_B T}{L^2 2\eta q} \frac{\tau_q^3}{t^2} \left(\frac{t}{\tau_q} + \exp(-t/\tau_q) - 1 \right)}_{\text{spectrum averaged over acquisition time}} \end{aligned} \quad (1.3)$$

with the length of the contour section a , $L = 2\pi R$, the vesicle radius R , absolute temperature T , viscosity of the surrounding medium η , camera integration/acquisition time t , $q = \sqrt{q_x^2 + q_y^2}$ and correlation time $\tau_q = (4\eta q)/(\sigma q^2 + \kappa q^4)$. Since this formula is derived for a flat membrane, it will deviate from the fluctuation spectrum of a closed, spherical vesicle, but only for the very lowest modes, which are long enough to feel the curvature of the vesicle (67). Fitting this expression to experimentally

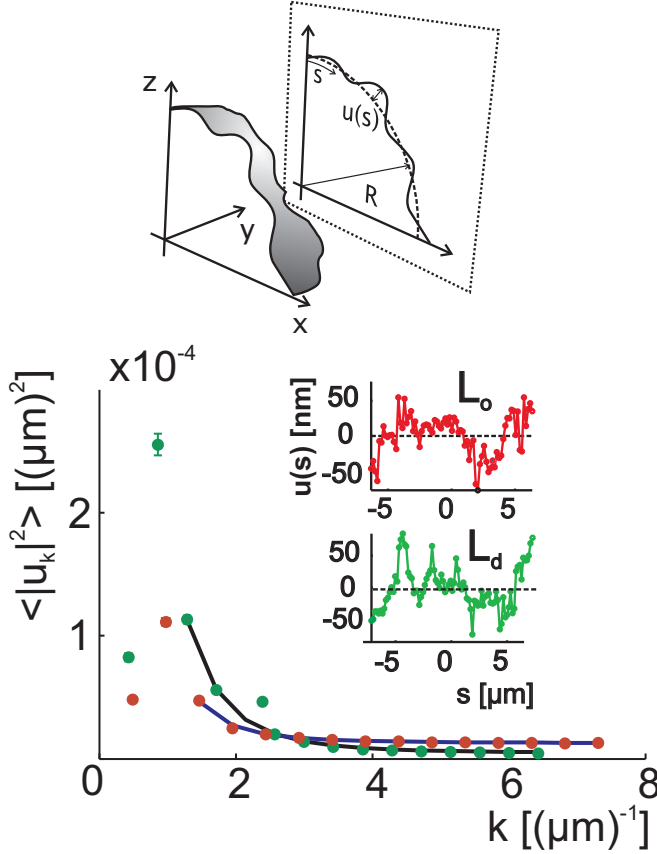


Figure 1.6

Top: The vesicle is projected along the y -axis on the plane of the camera (x - z plane). Fluctuations of the contour $u(s)$ are measured relative to the mean radius R of the approximately spherical vesicle poles. s is the arclength along the mean contour. Down: Typical experimental fluctuation spectra for the L_o phase (red dots) and the L_d phase (green dots) with fits of the theoretical expression for the spectrum, Eq. 1.3 (solid lines). Inset: Typical contour fluctuations.

determined fluctuation spectra for the two different phases reveals that the L_o phase is stiffer than the L_d phase (20) (Chap. 2 of this thesis), see Fig. 1.6. By detailed analysis of vesicle shapes, all membrane parameters can be determined (20, 71) (Chap. 2 of this thesis) including line tension and the difference in Gaussian bending moduli between the two phases. Alternatively, domain boundary fluctuations can be used to retrieve the

line tension (22, 84). Consistently, line tension around $1pN$ are found far away from the critical point where domain boundary fluctuations are absent. Close to the critical point, where domain boundaries fluctuate visibly, τ drops to $\approx 0.1pN$ and vanishes at the critical point. These values for the line tension suggest that small domains are stable against budding (85) and, taking into consideration active membrane recycling (86), domains in living cells should be at most $\approx 10nm$ in diameter (20) (Chap. 2 of this thesis).

Membrane mediated interactions Although complete phase separation is the ground state of a heterogeneous vesicle, domains with long term stability have been observed experimentally (70, 74, 87–89) (Chap. 3 of this thesis). Domain coalescence is kinetically hindered (88) which suggests an interaction between domains. As trapped coarsening is only observed in vesicles with budded domains, see Fig. 1.7, membrane mediated interactions (53–55) must be responsible for the observed effect.

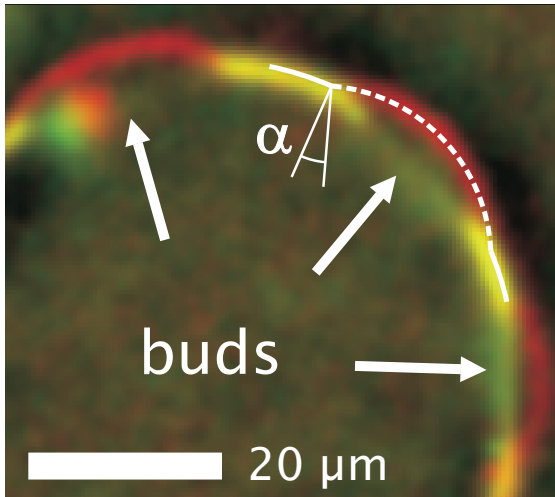


Figure 1.7

L_o buds (red) in a *L_d* background (yellow/green). α denotes the contact angle between the bud and the surrounding membrane.

This type of interactions is not restricted to membrane domains but also plays an important role for the organization of proteins (42, 53). Vesicles with bulged domains offers the unique possibility to study membrane mediated interactions exclusively, which would not be possible with proteins, due to their small size.

If the inter-domain forces are treated as harmonic springs, the spring

constant turns out to be $\approx 1k_B T/\mu m^2$ (89) (Chap. 3 of this thesis), see Fig. 1.8.

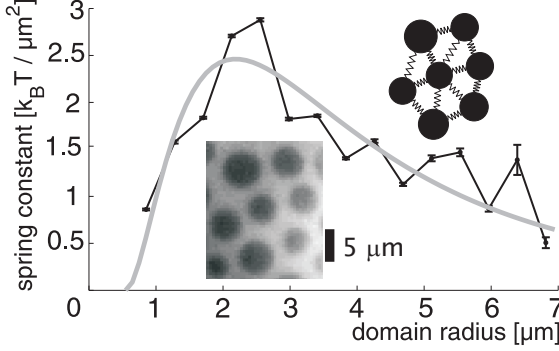


Figure 1.8

Spring constant k with respect to domain radius d (solid circles). The gray solid line shows a fit of Eq. 1.5 to the data. Left inset: L_O domains (dark) in an L_d background (bright). Right inset: Hookean spring model for domain interactions.

This value explains the long term stability of bulged domains (88) and their long range order. Membrane mediated interactions strongly depend on the strength of the distortions which are imposed by the inclusions (54). Consequently, the spring constant varies with domain size: while it first increases with domain radius, reflecting increased distortion of the environment, it decreases for larger radii due to an increased distance to neighboring domains (89) (Chap. 3 of this thesis). Below a certain critical size ($< 0.5\mu m$), the force vanishes. This critical size is set by the invagination length $\xi = \kappa/\tau$ which gives the length scale where interfacial and bending energy are of the same magnitude (90, 91). In domains that are small compared to ξ the line tension cannot push out the domain against the bending rigidity and the domain stays 'flat'. In this shape it does not distort its environment and causes no interactions. Due to this effect, domains grow by coalescence until they reach the critical size. Domain fusion then becomes kinetically hindered which results in a preferred domain size (89) (Chap. 3 of this thesis), which grows only slowly (88).

To describe the system quantitatively, bulged L_O domains can be treated, to first approximation, as spherical inclusions which locally distort the surrounding membrane (54) and thereby cause and experience membrane mediated interactions (89) (Chap. 3 of this thesis). Since the L_O domains are much stiffer than the surrounding membrane in the L_d phase ($\frac{\kappa_O}{\kappa_d} \approx 4$ (20), Chap. 2 of this thesis), it is a good approximation to consider them rigid. The interaction potential between two rigid inclusions in an infinite, asymptotically flat membrane was calculated in

(54):

$$V = 4\pi\kappa_d(\alpha_1^2 + \alpha_2^2) \left(\frac{a}{r}\right)^4 \quad (1.4)$$

where r is the distance between the two inclusions, a is a cutoff length scale (typically the membrane thickness, $\approx 4\text{nm}$), α_1 and α_2 are the domains' contact angles with the surrounding membrane (see Fig. 1.7) and κ_d is the bending modulus of the surrounding L_d phase. This result should approximately hold also for domains on a spherical vesicle if the domain radius is small compared to the radius of the vesicle. In general a domain is surrounded by many others. Therefore it is reasonable to assume that the domain moves in a harmonic potential with spring constant k . If the contact angle α and average domain distance grow linearly with domain size d , the dependence of k on d is given by:

$$k(d) = A \frac{(d - d_0)^2}{(r_0 + cd)^6}. \quad (1.5)$$

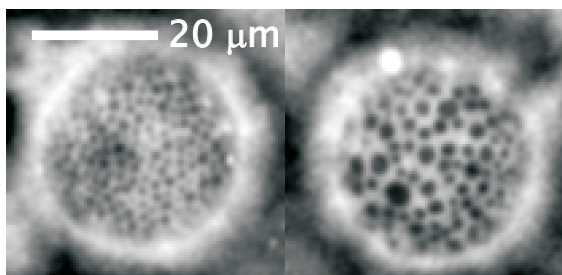
where r_0 and c are parameters which can be determined directly from the raw data and A and d_0 are fit parameters. Fig. 1.8 shows a fit of this expression to experimental data. The point d_0 , where the interaction vanishes, is found to be close to the invagination length ξ (89) (Chap. 3 of this thesis).

Membrane mediated interactions are not necessarily repulsive. In coarse grained simulations, membrane mediated interactions induced the aggregation of small rigid caps (53). This suggests that the magnitude (and sign) of membrane mediated interactions depend on the length scale and membrane material properties.

Interestingly, it was found that membrane mediated interactions sort domains by size (56) (Chap. 4 of this thesis), see Fig. 1.9 Since big domains repel more strongly than small domains the total energy can be lowered by increasing the vesicle area covered by big domains. This can be achieved only if domains are sorted by size (56) (Chap. 4 of this thesis). Translated to proteins this might provide a mechanism for creating cell polarity or organize proteins on a large scale.

1.3 Living cells

The properties of a complex system can only be determined by examining the system itself: membrane heterogeneity must be studied ulti-

**Figure 1.9**

Two sides of the same phase separated vesicle. The L_d phase is fluorescently stained. The (dark) L_o domains in the two different regions show a marked difference in size.

mately in living cells. Processes during cell death, methods of cell fixation or preparation of cell extracts all potentially induce formation of domains. Since heterogeneities exist both on small length and time scales, advanced experimental methods capable of resolving these scales must be employed. With FRET (Fluorescence Resonance Energy Transfer), for instance, spatial proximity between molecules in the nanometer regime can be measured (92), even at a single-molecule level (93). This technique is, however, limited to very small distances (typically $< 10\text{nm}$). Recently developed superresolution techniques (94) also deliver the necessary spatial resolution but suffer from a low acquisition speed. Up to now only techniques based on single molecule fluorescence or single particle tracking can address relevant length and time scales (95).

Single molecule techniques (96) have become a major tool for biophysics (97) and, lately, systems biology (98–100). The reason for that is twofold: first, single-molecule events can have biological relevance themselves (101) and second, the behavior of single molecules is a very sensitive readout for the characteristics of big ensembles. For instance, FRAP (Fluorescence recovery after photobleaching) is a well established technique to determine diffusion coefficients (102). However, FRAP averages over many molecules, which makes extensive modeling necessary to interpret the results (103). Here we discuss how signatures of membrane micro structure can be identified in single molecule tracking experiments.

1.3.1 Single molecule tracking

Single molecule tracking is based on the detection of the scattered or fluorescence light coming from a single particle or molecule that is coupled

to the molecule of interest. A successful experiments depends on the following key points: 1. A suitable probe has to be chosen that is both minimally perturbing and gives a sufficient signal. 2. The optical detection scheme has to be sensitive enough to detect the single probe and deliver the desired time resolution. Besides, photo destruction of the cell must be minimized. 3. The positions of the probe have to be determined with high, sub-diffraction resolution. 4. The kinetic parameters must be extracted from the molecule positions by constructing trajectories or analyzing directly spatio-temporal correlations. Besides the positions, the intensities of diffraction limited spots can be used to measure molecule numbers in these spots.

Probes Useful probes differ greatly in size, biocompatibility and photophysical properties. Gold particles provide an excellent signal to noise ratio and unlimited observation time, since their detection is based on light scattering. Gold is nontoxic and well-known surface chemistry allows them to be coupled to a wide range of molecules. The labeling ratio is, however, rather undefined and unspecific binding is an issue (36). Fluorescent quantum dots are considerably smaller than gold beads and possess good photostability (104, 105) though blinking might be a problem for some applications. The emission wavelength of quantum dots changes with their size which results in a broad spectrum of available colors. Quantum dots have to be coated to render them biocompatible and a one-to-one labeling ratio is difficult to achieve. Artificial dye molecules, like Cy5, are small but suffer from photobleaching. They can be coupled to molecules of interest by covalent bonds which guarantees monomeric labeling. For the study of proteins in living cells autofluorescent proteins are a good compromise between the probes discussed so far. They are intrinsically biocompatible and one-to-one labeling is easily achieved by fusing them to the protein of interest, which allows quantitative assessment of protein stoichiometry (106–108) (Chap. 7 of this thesis). Fluorescent proteins exist in different colors, which enables multiplexing. In contrast to all other probes also intracellular structures can be stained in a minimally invasive way. The biggest disadvantage of fluorescent proteins is their complex photophysics (109), especially the poor photostability (110). However, as we discuss below, improved data analysis techniques and optimization of experimental parameters can alleviate many of the problems related to photophysics (108, 111) (Chaps. 5 and 7 of this thesis). Recently, pho-

toactivatable fluorophores have been used to increase the number of signals which can be collected on a single cell (112).

Optical detection scheme Single molecules are usually detected with a microscope in widefield (113–116) or total internal reflection (117, 118) configuration. More elaborate ways of illumination have been developed to diminish background and minimize photodestruction (119, 120). A CCD camera is most commonly chosen as detection device. Their advantages comprise a high quantum yield, low noise and ability to image the whole field of view at once. A fluorescence signal coming from a single-molecule is described completely by the following parameters: intensity, spatial extension and polarization (or anisotropy), emission spectrum and, most importantly, position. All of these parameters contain relevant information about the molecule of interest and its local environment. As we discuss below, fluorescence intensity can be used to determine the stoichiometry of a molecule complex or assess membrane heterogeneity (108, 121) (Chap. 7 of this thesis). Anisotropy contains information about rotational diffusion of the probe (122, 123) and structural information (124). A shift of the emission spectrum reports changes in the local environment, e.g. the packing of lipids, if the used fluorescent probe is sensitive to those (125). Most importantly, the position of a fluorescent probe can be determined with a very high positional accuracy, down to a few *nm* (95). With the help of several focal planes (126) or by introducing an astigmatism (127), molecules can be localized in 3D.

Position determination Among the several methods to determine a molecule's position in 2D, fitting to the point spread function of the microscope is the most reliable procedure (128), see Fig. 1.10. The achievable positional accuracy depends on the signal to noise ratio (129, 130) and is typically 20–40 *nm* for fluorescent proteins in living cells (131). In fixed cells or with sufficient data acquisition speed, the single molecule positions can be combined to a high resolution image of the cell (94), a direct readout of heterogeneity. However, the lack of high temporal resolution conceals transient processes. Those processes manifest themselves in anomalous diffusion of single molecules addressed by single-molecule or single-particle tracking experiments (35, 36, 131–133). The achievable temporal resolution is typically a few *ms* for experiments in living cells (36, 131).

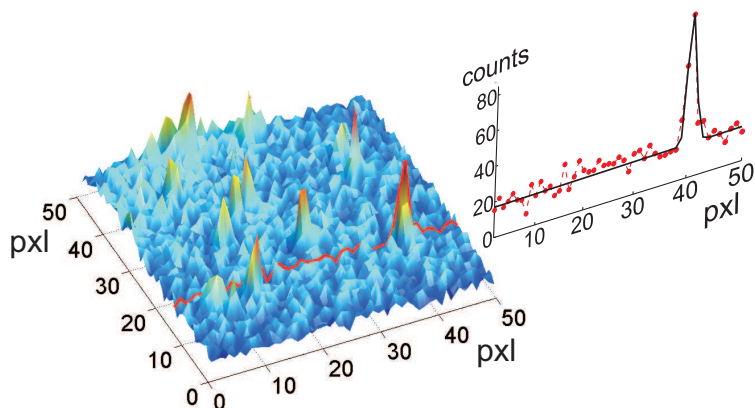


Figure 1.10

Left: Fluorescence signals coming from single molecules obtained in a live cell, right: 1D example for peak fitting. A gaussian (black) is fitted to the raw data (red) to determine intensity and position. pxl = 220 nm

Determination of kinetic parameters, PICS The recorded molecule positions are typically connected to trajectories (134–136) from which kinetic parameters are retrieved. The reconstruction of trajectories is, however, difficult, if the used fluorophore suffers from excessive blinking. Additionally, if the photostability is low, trajectories are short, which results in a big error in the calculated diffusion coefficients (137). Alternatively, kinetic parameters can be retrieved directly from spatio-temporal correlations of single molecule positions by Particle Image Correlation Spectroscopy (PICS) (111) (Chap. 5 of this thesis). This method is based on image correlation techniques (138) but exploits the high temporal and spatial resolution of single molecule tracking. In contrast to conventional tracking algorithms no *a priori* knowledge about diffusion speeds is required. PICS works for high molecule densities which makes it the ideal analysis method for experiments with photoactivatable fluorophores (112). Blinking and bleaching do not bias this method since uninterrupted trajectories are not required to determine mean squared displacements (MSDs) and eventually diffusion coefficients in a robust way. On the contrary, long lived dark states or reversible bleached states actually extend the accessible observation period compared to conventional tracking methods. In PICS a simple algorithm is used to determine the cumulative distribution

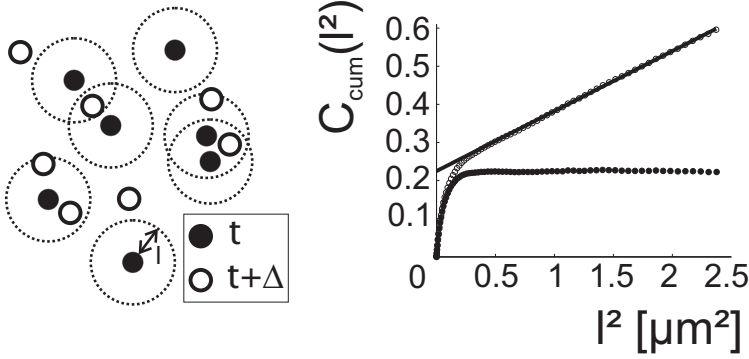


Figure 1.11

Left: PICS algorithm: count all molecules at time t_2 (open circles) which are closer than l (dashed circle) to a molecule at time t_1 (solid circles). Averaged over space and time this results in the cumulative correlation function $C_{\text{cum}}(l, \Delta t)$ shown on the right (open circles). Subtraction of random correlations (solid line) results in the cumulative probability $P_{\text{cum}}(l, \Delta t)$ apart from a normalization factor (solid circles).

function $P(l, \Delta t)$ for the length l of diffusion steps during the time lag Δt .

As shown in Fig. 1.11, the amount of molecules at time $t + \Delta t$ is counted that is closer than l to another molecule at time t . By subtraction of the linear contribution at bigger l - which stems from accidental proximity of uncorrelated molecules - $P_{\text{cum}}(l, \Delta t)$ is obtained. If the diffusion coefficient D is determined directly from $P_{\text{cum}}(l, \Delta t)$, assuming normal diffusion, the relative error is

$$\frac{\Delta D}{D} = \frac{1}{\ln 2} \sqrt{\eta^2 + (1 - (1/2)^{2/N})} \quad (1.6)$$

where N is the number of diffusion steps (= number of molecules per image $\langle m \rangle \times$ number of image pairs M) and

$$\eta = \sqrt{16\pi \ln 2 \frac{cD\Delta t}{M}} \quad (1.7)$$

with the concentration of molecules c , diffusion coefficient D , time lag Δt and recorded image pairs M . While the first term under the root in Eq. 1.6 (η^2) is caused by the method, the second term is unavoidable and due to the stochastic nature of diffusion. Interestingly, the scaling behavior of η shows that the molecules can in principle be very dense and diffuse very

quickly on the time scale of a time lag Δt . If enough image pairs M are recorded, the error due to the method will be small.

To extract the MSD with respect to time lag Δt a model for the cumulative distribution function $P_{\text{cum}}(l, \Delta t)$ has to be fitted. For a homogeneous population of molecules diffusing normally the expected cumulative distribution function is (139)

$$P_{\text{cum}}(l, \Delta t) = 1 - \exp\left(-\frac{l^2}{4D\Delta t}\right) \quad (1.8)$$

This functional form very often does not give a good fit to the experimental data, since the behavior of the observed molecules is in general very heterogeneous. A population of molecules which exhibits two different diffusion coefficients D_1 and D_2 is represented by the following distribution function (132)

$$P_{\text{cum}}(l, \Delta t) = \alpha \left(1 - \exp\left(-\frac{l^2}{4D_1\Delta t}\right)\right) \quad (1.9)$$

$$+ (1 - \alpha) \left(1 - \exp\left(-\frac{l^2}{4D_2\Delta t}\right)\right) \quad (1.10)$$

where α is the fraction of the molecules diffusing with D_1 . Naturally, this distribution fits better than the distribution for a single fraction. However, the biological nature of the two fractions has to be determined carefully from the biological context on a case by case basis.

$P(l, \Delta t)$ can also be compared to distribution functions created by Monte Carlo simulations (140). The disadvantage of PICS is the loss of individual trajectories but the diffusion parameters obtained by PICS in an unbiased way can be used as initial parameters for elaborate tracking algorithms (134).

Analysis of fluorescence intensities Arguably the second most important parameter of a fluorescence signal is its intensity. By measuring the intensity stemming from a diffraction limited spot, the number of fluorophores can be determined. In this way it is possible to determine stoichiometry in biochemical reactions, count protein subunits (106) or quantify protein clustering due to heterogeneity (108) (Chap. 7 of this thesis). In single molecule experiments intensity is used in two ways to determine fluorophore numbers: the number of bleaching steps in multistep photobleaching is counted (106) or histograms of fluorophore intensities

are evaluated (107, 108, 121, 141) (Chap. 7 of this thesis). The complex photophysics, especially blinking, of autofluorescent proteins render both approaches difficult. Fluorescence traces do not show clear bleaching steps and intensity distributions are very broad, which obscures differences between e.g. monomers and dimers. However, it was shown that, with the right choice of experimental parameters, intensity histograms can be used for robust assessment of single-molecule stoichiometries (107, 108) (Chap. 7 of this thesis). If a fluorophore is illuminated so long that it bleaches during the illumination time, semi-classical Mandel theory predicts for the intensity distribution $p(n)$ (108) (Chap. 7 of this thesis)

$$p(n) = \frac{1}{N} \left(1 + \frac{1}{N}\right)^{-(n+1)} \quad (1.11)$$

where N is the mean number of photons detected. This prediction is readily verified in experiments (108) (Chap. 7 of this thesis), see Fig. 1.12. The intensity distributions of multimers can be derived by convolution of

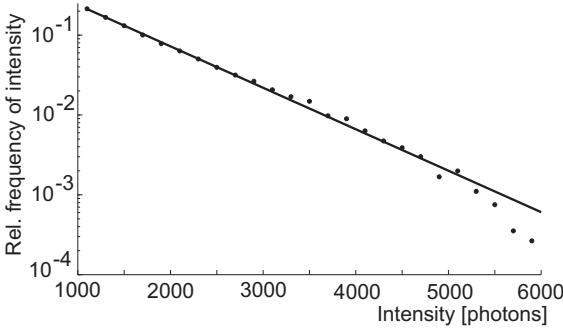


Figure 1.12

Logarithm of experimental intensity distribution of eYFP in living cells. The illumination time was 50 ms at an intensity of 3 kW/cm². A one-parameter fit of Eq. (1.11) (solid line) to the experimental intensity distribution for single YFPs (circles) gives $N = 837 \pm 3$ photons.

the monomer intensity distribution, Eq. 1.11, with itself (121).

Intensity histograms are also influenced by the thresholding procedure needed to separate signals from noise. Typically, the raw data is filtered to increase the signal to noise ratio. The matched filter principle (142) prescribes a filter that resembles the signal. For peaks with a width w , the raw images are therefore filtered with a Gaussian of width w . A peak in the filtered image is considered real if its intensity exceeds the noise level σ by a factor of t . The probability to detect a peak with width w and integrated intensity A at a noise level σ can be shown to be (108)

(Chap. 7 of this thesis)

$$p_{\text{det}}(A; \sigma, w, t) = \frac{1}{2} \left(1 + \operatorname{erf} \left(\frac{A}{\sqrt{8\pi\sigma w}} - \frac{t}{\sqrt{2}} \right) \right) \quad (1.12)$$

Consequently, the measured intensity distributions are a product of the real intensity distribution of the molecule and Eq. 1.12. If the influence of the detection probability is properly taken into consideration, measured intensity distributions faithfully reflect underlying stoichiometry (108) (Chap. 7 of this thesis). To ensure that labeling stoichiometry is preserved, this method can be combined with the TOCCSL (Thinning out clusters while conserving stoichiometry of labeling) illumination scheme (143).

1.3.2 Selected results

GPCR signaling G protein coupled receptors, the biggest subfamily of membrane receptors, are a major drug target (144). Membrane heterogeneity is supposed to be involved in GPCR desensitization and internalization (13) and suspected to influence the kinetics of the signaling cascade (145). In the canonical model for GPCR signaling a ligand binds to the receptor on the outside of the cell, which, as a reaction, changes the conformation of its cytosolic part, see Fig. 1.13. This activation of the receptor enables it to interact with its G protein which may or may not be precoupled to the receptor. The G_α subunit of the G protein dissociates after interaction with the receptor and engages in downstream signaling. The activation of the G protein happens very quickly after ligand binding (146), which suggests that the receptor and its G protein are localized in micro domains or that they are coupled even in the absence of a ligand. Experiments addressing this precoupling give contradictory results (145, 147, 148). Application of Particle Image Correlation Spectroscopy to Adenosine A_1 receptor signaling revealed the influence of membrane heterogeneity and precoupling (11) (Chap. 6 of this thesis).

First of all, the dynamics of the Adenosine A_1 receptor is well described by a two-fraction model, Eq. 1.9. With the help of stimulation and decoupling experiments, the slow fraction was found to comprise receptors that interact with a G protein. Both the slow and the fast fraction show anomalous diffusion, see Fig. 1.14. This is evident from a non-linear dependence of the mean squared displacement on time. Normal diffusion,

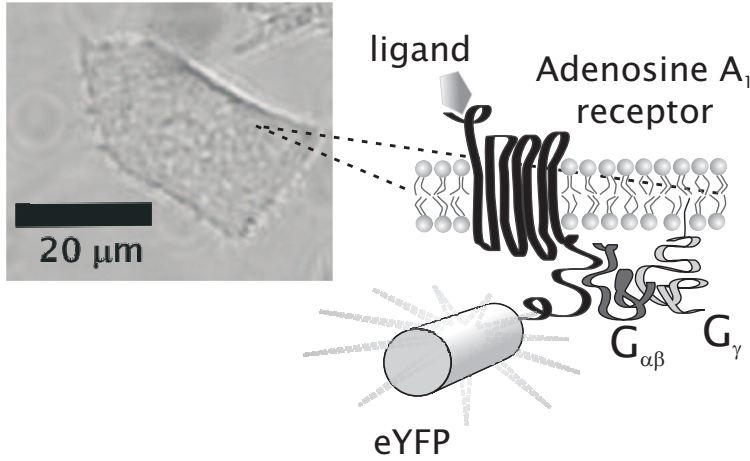


Figure 1.13

Left: Brightfield image of a living CHO cell transfected with the construct shown on the right. Right: G protein coupled receptor with its G protein which consists of three subunits α , β and γ . The receptor is tagged by eYFP fused to the C-terminus.

by contrast, is characterized by an MSD that increases linearly with time lag. In two dimensions the MSD is given by

$$\text{MSD}_{\text{normal}}(\Delta t) = 4D\Delta t \quad (1.13)$$

with a diffusion constant D .

Non-linear behavior can be introduced by membrane micro structure which interferes with the movement of the molecule. If the molecule is confined to a finite region of size L the MSD asymptotically becomes constant (29)

$$\text{MSD}_{\text{confined}}(\Delta t) = \frac{L^2}{3} \left[1 - \exp\left(-\frac{12D\Delta t}{L^2}\right) \right] \quad (1.14)$$

In the case of the Adenosine A_1 receptor, the slope of the MSD decreases for bigger time lags but stays finite. The receptor is not simply confined, its dynamics is better described by walking diffusion (133). In this model a molecule diffuses with diffusion coefficient D_{micro} within a domain of size L and the domain itself diffuses normally with diffusion coefficient D_{macro} , see Fig. 1.14. Consequently, the MSD of the molecule is the sum of the

MSD for normal and confined diffusion

$$\text{MSD}_{\text{walking}}(\Delta t) = \frac{L^2}{3} \left[1 - \exp \left(-\frac{12D_{\text{micro}}\Delta t}{L^2} \right) \right] + 4D_{\text{macro}}\Delta t \quad (1.15)$$

This functional form of the MSD can also arise in a different situation: In the hopping diffusion model a molecule is transiently confined to a domain with size L , diffusing with coefficient D_{micro} , see Fig. 1.14. Infrequently, the molecule hops to a neighboring domain which results in a smaller effective diffusion coefficient D_{macro} on length scales that are big compared to L . By fitting of Eq. 1.15 to experimental data for the Adenosine A_1 receptor D_{micro} , D_{macro} and L can be retrieved, see Fig. 1.14. Stimulation of the receptor with an agonist results in a decrease of the fast fraction. In other words, receptors translocate to microdomains with higher membrane viscosity upon ligand binding. These domains depend on the cytoskeleton, since they are not present in cell blebs. Probably, the cortical actin fosters the formation of membrane heterogeneities, like e.g. clathrin coated pits or caveolae. Additionally, it was shown that a fraction of the receptors is precoupled to the G protein: the fast fraction increases after decoupling of receptor and G protein. Probably, the G protein mediates the interaction with micro domains. In summary, micro domains as well as precoupling play crucial roles in Adenosine A_1 receptor signaling.

Clustering of H-Ras Another membrane protein which has been shown to translocate to microdomains upon activation is the small GTPase H-Ras (131). Ras proteins are involved in cell growth, proliferation and differentiation. The various isoforms, N-Ras, K-Ras and H-Ras activate several effectors to different extents. This specificity was speculated to depend on membrane domain localization since K-Ras and H-Ras mainly differ in their membrane-anchoring region. This notion was confirmed by electron microscopy studies (149), which showed that K-Ras and H-Ras membrane anchors are localized in distinct membrane domains that are tens of nanometers in size. FRET experiments show that Ras activation - i.e. binding of GTP- results in a decrease in mobility (93). A single particle study on constitutively active and inactive mutants of H-Ras showed, that a fraction of the active mutant is localized in micro domains (131). This confinement explains the decrease in mobility upon activation. Recently, this result was confirmed with the PICS method (111) (Chap. 5 of this

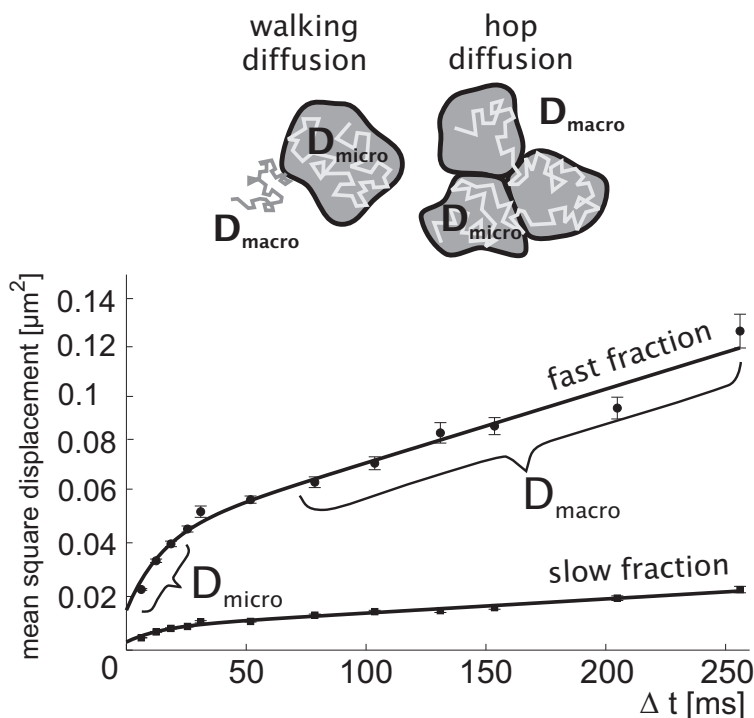
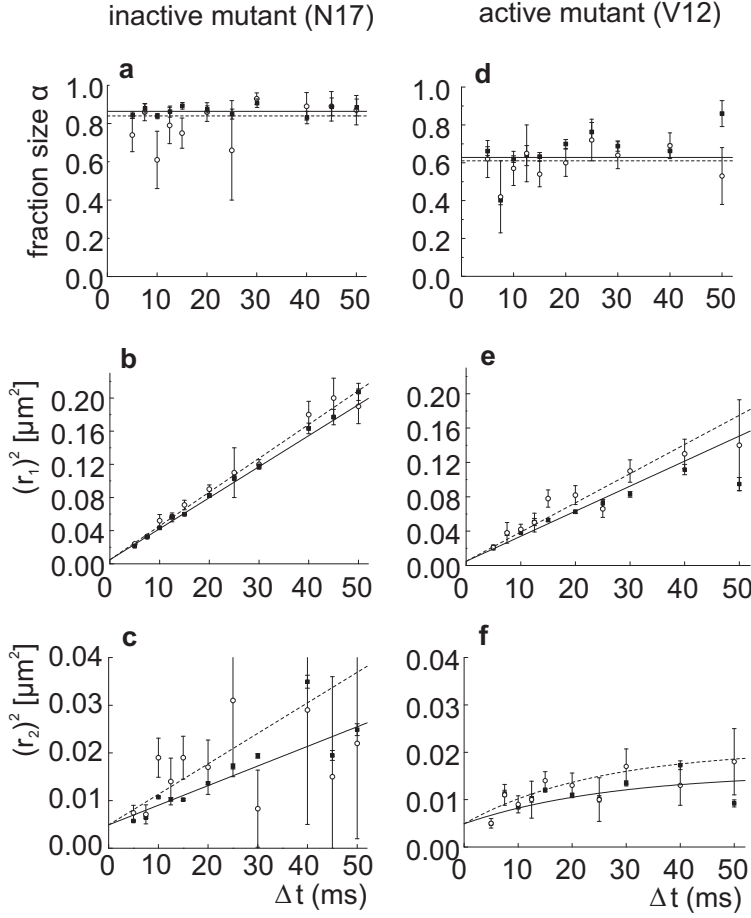


Figure 1.14

Mean square displacements of the Adenosine A_1 receptors in living CHO cells. The fast receptor fraction is about 70 %. A fit to the walking diffusion model (solid line) yields for the fast fraction $D_{\text{micro}} = 0.47 \pm 0.12 \mu\text{m}^2/\text{s}$, $D_{\text{macro}} = 0.07 \pm 0.01 \mu\text{m}^2/\text{s}$, $L = 287 \pm 20 \text{ nm}$ and for the slow fraction $D_{\text{micro}} = 0.10 \pm 0.02 \mu\text{m}^2/\text{s}$, $D_{\text{macro}} = 0.01 \pm 0.001 \mu\text{m}^2/\text{s}$, $L = 129 \pm 7 \text{ nm}$. Inset: illustration for walking and hop diffusion. Both lead to an MSD with a bigger slope on small time scales (D_{micro}) and a smaller slope on long time scales (D_{macro}).

thesis), see Fig. 1.15. Interestingly, the membrane anchor of H-Ras alone is found to localize in micro domains (150). Clustering of this membrane anchor was recently confirmed by a different method (108) (Chap. 7 of this thesis): analysis of the intensity histograms of YFP labeled anchors, as described above, revealed that they are found in clusters which cannot be explained by a random homogeneous distribution, see Fig. 1.16.

**Figure 1.15**

Diffusional behavior of HRas. Fraction α (a & d) and mean square displacements r_1^2 (b & e) and r_2^2 (c & f) as functions of Δt for the constitutive inactive (N17) (a-c) and the constitutive active (V12) mutant (d-f) of HRas. The slow fraction of the active mutant (f) exhibits confined diffusion. Open circles / dashed lines correspond to conventional tracking results (131); solid squares / solid lines to results obtained by the PICS method. In the case of the conventional tracking errorbars correspond to the error of the fitting of the two fraction model, for PICS the size of the errorbars is given by Eq. 1.6.

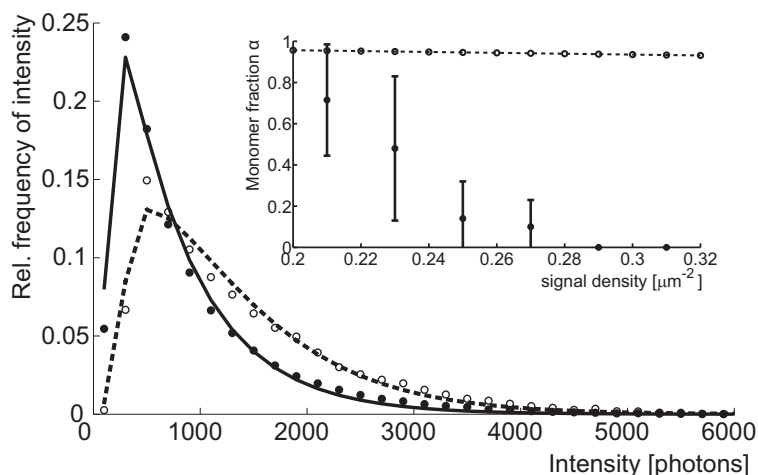


Figure 1.16

Intensity histograms of YFP labeled H-Ras membrane anchors. The solid circles correspond to a situation with only monomeric anchors (low molecule density). The open circles show the results for a higher peak density ($\rho = 0.25 \mu\text{m}^{-2}$). The shift to higher intensities with increased density is due to the increased number of molecule dimers and higher multimers. The solid and dashed lines are fits to theoretical expressions for single-molecule intensity distributions. Inset: Fraction of monomers of the H-Ras membrane anchor in living CHO cells (circles). The solid line shows the theoretically expected monomer fraction for a uniform distribution of molecules.

1.4 Future directions

Experiments on living cells and artificial membranes have inspired and stimulated each other in the passed years and they will continue to do so. The challenge for the future is to bring both approaches closer together and the results to ever better and more quantitative agreement. Artificial membranes must be produced that resemble their living counterparts more closely while the necessary increase in complexity must not compromise the most important property of artificial systems: their controllability. Experiments on live cells, on the other hand, have to become more controlled and at the same time less invasive. Much progress can be made by developing ways to manipulate cells more specifically instead of using drugs that have many - sometimes unknown - effects. But improvements in both directions will be worthwhile since there are many open questions left.

If the membrane turns out to be indeed close to a miscibility phase transitions also *in vivo* the question is whether composition fluctuations are persistent enough to segregate proteins. Only then they could fulfill a significant biological function. To answer this it might be worthwhile to study the diffusion of peptides and proteins in GUVs (151, 152) that show strong composition fluctuations.

The nature of the coupling between the cytoskeleton and the membrane is still to be unraveled. Models range from indirect interactions via molecule pickets (30) to direct, mechanical coupling (31, 34). Only careful experiments in a clearly defined system can decide between these competing views. Those experiments start to be feasible due to recent progress on *in vitro* reconstitution of an actin cortex (153) and *in vitro* translation (154), both in GUVs. Models for the interaction between cytoskeleton and membrane predict asymmetric diffusion if the cytoskeleton is stretched (34). Stretching of the cell can be caused by a gradient in stiffness of the surrounding medium or simply during cell locomotion. The coupling of an asymmetric cell shape to non-isotropic diffusion might be important in processes like directional sensing and chemotaxis.

Concerning membrane mediated interactions, there are already quite a few *in vivo* experiments that showed a significant role of curvature in biological processes (45, 46) and more effects are predicted (155). The relation between membrane mechanics and biological processes, like signaling, is therefore likely to attract even more attention (156).

These questions and exciting technological developments conceived to answer them make membrane heterogeneity a lively field of research for years to come.

CHAPTER 2

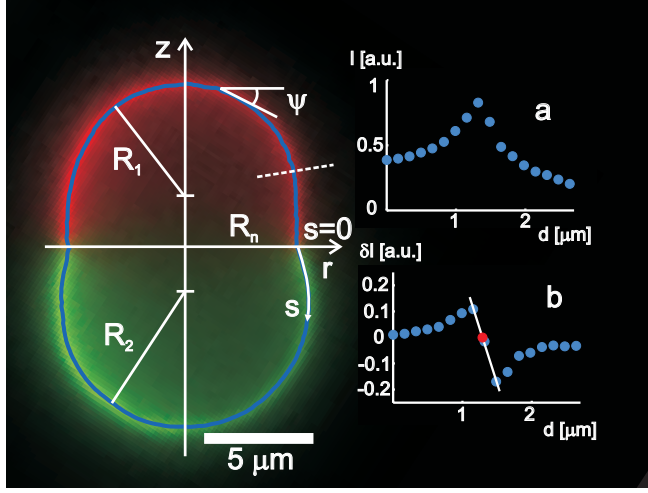
ACCURATE DETERMINATION OF ELASTIC PARAMETERS FOR MULTI-COMPONENT MEMBRANES¹

Heterogeneities in the cell membrane due to coexisting lipid phases have been conjectured to play a major functional role in cell signaling and membrane trafficking. Thereby the material properties of multiphase systems, such as the line tension and the bending moduli, are crucially involved in the kinetics and the asymptotic behavior of phase separation. In this Letter we present a combined analytical and experimental approach to determine the properties of phase-separated vesicle systems. First we develop an analytical model for the vesicle shape of weakly budded biphasic vesicles. Subsequently experimental data on vesicle shape and membrane fluctuations are taken and compared to the model. The combined approach allows for a reproducible and reliable determination of the physical parameters of complex vesicle systems. The parameters obtained set limits for the size and stability of nanodomains in the plasma membrane of living cells.

¹This chapter is based on: S. Semrau, T. Idema, T. Schmidt, C. Storm, Accurate determination of elastic parameters for multi-component membranes, *Phys. Rev. Lett.*, **100**, 088101, (2008)

2.1 Introduction

The recent interest in coexisting phases in lipid bilayers originates in the supposed existence of lipid heterogeneities in the plasma membrane of cells. A significant role in cell signaling and traffic is attributed to small lipid domains called “rafts” (8, 19, 38, 131, 157, 158). While their existence in living cells remains the subject of lively debate, micrometer-sized domains are readily reconstituted in giant unilamellar vesicles (GUVs) made from binary or ternary lipid mixtures (60). Extensive studies of these and similar model systems have brought to light a rich variety of phases, phase transitions and coexistence regimes (73). In contrast to these model systems, no large (micrometer sized) membrane domains have been observed *in vivo*. If indeed phase separation occurs *in vivo*, additional processes which can arrest it prematurely must be considered. It has been suggested that nanodomains might be stabilized by entropy (159) or that, alternatively, active cellular processes are necessary to control the domain size (86). A third explanation is that curvature-mediated interactions conspire to create an effective repulsion between domains, impeding and ultimately halting their fusion as the phase separation progresses. Each of these three processes depends critically on membrane parameters such as line tension (90), curvature moduli and even the elusive Gaussian rigidities (91). Although some studies report values (71) or upper bounds (160, 161) for these membrane parameters, a systematic method to determine them from experiments that does not require extensive numerical simulation and fitting is lacking. We present here a straightforward fully analytical method that allows for a precise, simultaneous determination of the line tension, the bending rigidity and the difference in Gaussian moduli from biphasic GUVs. Both the liquid ordered L_o and the liquid disordered L_d phase are quantitatively characterized with high accuracy. Our method relies on an analytical expression for the shape of a moderately budded vesicle. A one-parameter fit to experimental shapes permits unambiguous determination of the line tension and the difference in Gaussian moduli. Our results provide important clues as to the origin and magnitude of long-ranged membrane-mediated interactions, which have been proposed recently as an explanation for the trapped coarsening (53, 88) and the very regular domain structure of a meta-stable state (70) found in experiments. Furthermore, our results show that nanometer-sized phase separated domains will be stable in life cells.

**Figure 2.1**

Fluorescence raw data (red: L_o domain, green: L_d domain) with superimposed contour (light blue). Insets: principle of contour fitting; a: intensity profile normal to the vesicle contour (taken along the dashed line in the main image); b: first derivative of the profile with linear fit around the vesicle edge (white line). The red point marks the vesicle edge.

2.2 Model

The free energy associated with the bending of a thin membrane is described by the Canham-Helfrich free energy (81, 82). We ignore any spontaneous curvature of the membrane because the experimental system has ample time to relax any asymmetries between the leaflets. For a two-component vesicle with line tension τ between the components, the free energy then reads:

$$\mathcal{E} = \sum_{i=1,2} \int_{S_i} \left(2\kappa_i H^2 + \kappa_G^{(i)} K + \sigma_i \right) dA - pV + \tau \oint_{\partial S} d\ell, \quad (2.1)$$

where the κ_i and $\kappa_G^{(i)}$ are the bending and Gaussian moduli of the two phases, respectively, the σ_i are their surface tensions, and p is the internal pressure. In equilibrated shapes such as our experimental vesicles, the force of the internal Laplace pressure is compensated by the surface tensions; consequently, both contributions drop out of the shape equations (162–164). For each phase, we integrate the mean (H) and Gaus-

sian (K) curvature over the membrane patch S_i occupied by that phase; the line tension contributes at the boundary ∂S of the two phases. Using the Gauss-Bonnet theorem, we find that the Gaussian curvature term yields a constant bulk contribution plus a boundary term (165).

The axisymmetric shapes of interest (Fig. 2.1) are fully described by the contact angle ψ as a function of the arc length s along the surface contour. The coordinates $(r(s), z(s))$ are fixed by the geometrical conditions $\dot{r} = \cos \psi(s)$ and $\dot{z} = -\sin \psi(s)$, where dots denote derivatives with respect to the arclength. Variational calculus gives the basic shape equation (162–164):

$$\ddot{\psi} \cos \psi = -\frac{1}{2} \dot{\psi}^2 \sin \psi - \frac{\cos^2 \psi}{r} \dot{\psi} + \frac{\cos^2 \psi + 1}{2r^2} \sin \psi. \quad (2.2)$$

This equation holds for each of the phases separately. The radial coordinate $r(s)$ and tangent angle $\psi(s)$ must of course be continuous at the domain boundary. Additionally, the variational derivation of equation (2.2) gives two more boundary conditions (166):

$$\lim_{\varepsilon \downarrow 0} (\kappa_2 \dot{\psi}(\varepsilon) - \kappa_1 \dot{\psi}(-\varepsilon)) = (\Delta\kappa + \Delta\kappa_G) \frac{\sin \psi_0}{R_n}, \quad (2.3)$$

$$\begin{aligned} \lim_{\varepsilon \downarrow 0} (\kappa_2 \ddot{\psi}(\varepsilon) - \kappa_1 \ddot{\psi}(-\varepsilon)) \\ = -(2\Delta\kappa + \Delta\kappa_G) \frac{\cos \psi_0 \sin \psi_0}{R_n^2} + \frac{\sin \psi_0}{R_n} \tau, \end{aligned} \quad (2.4)$$

with R_n and ψ_0 the vesicle radius and tangent angle at the domain boundary, $\Delta\kappa = \kappa_1 - \kappa_2$, $\Delta\kappa_G = \kappa_G^{(1)} - \kappa_G^{(2)}$, and the domain boundary located at $s = 0$.

The sphere is a solution of the shape equation (2.2); we can therefore use it as an ansatz for the vesicle shape far from the domain boundary. We split the vesicle into three parts: a neck domain around the domain boundary, where the boundary terms dominate the shape, and two bulk domains, where the solution asymptotically approaches the sphere. Perturbation analysis, performed by expanding Eq. (2.2) around the spherical shape, then gives for the bulk domains:

$$\psi_{\text{bulk}}^{(i)}(s) = \frac{s + s_0^{(i)}}{R_i} + c_i R_i \log \left(\frac{s}{s_0^{(i)}} \right). \quad (2.5)$$

Here R_i is the radius of curvature of the underlying sphere and $s_0^{(i)}$ the distance (set by the area constraint on the vesicle) from the point $r = 0$ to

the domain boundary. As was shown by Lipowsky (90), the *invagination length*, defined as the ratio $\xi_i = \kappa_i/\tau$ of the bending modulus and the line tension, determines the size of the neck region. Our three-domain approach applies when this invagination length is small compared to the size of the vesicle. At $s = \xi_i$ the line tension, rather than the bending modulus, becomes the dominant term in the energy. Self-consistency of the solution requires that the deviation from the sphere solution at that point be small, *i.e.* given by the dimensionless quantity ξ_i/R_i . This fixes the integration constant c_i .

Near the domain boundary, ψ must have a local extremum in each of the phases and we can expand it as

$$\psi_{\text{neck}}^{(i)}(s) = \psi_0^{(i)} + \dot{\psi}_0^{(i)}s + \frac{1}{2}\ddot{\psi}_0^{(i)}s^2. \quad (2.6)$$

These neck solutions must match at the domain boundary and also satisfy conditions (2.3, 2.4). Furthermore, they also need to match the bulk solutions to ensure continuity of ψ and its derivative $\dot{\psi}$. In total this yields seven equations for the eight unknowns $\{\psi_0^{(i)}, \dot{\psi}_0^{(i)}, \ddot{\psi}_0^{(i)}, s_i\}$. The necessary eighth equation is provided by the condition of continuity of $r(s)$ at the domain boundary.

Put together the neck and bulk components of ψ give a vesicle solution for specified values of the material parameters $\{\kappa_i, \Delta\kappa_G, \tau\}$. This solution compares extremely well to numerically determined shapes (obtained using the Surface Evolver package (167), Fig. 2.4). Moreover, for the symmetric case of domains with identical values of κ , we can compare to earlier modeling in Ref. (91). The vesicle can then be described by a single dimensionless parameter $\lambda = R_0/\xi$, where $4\pi R_0^2$ equals the vesicle area. The ‘budding transition’ (where the broad neck destabilizes in favor of a small neck) is numerically found in Ref. (91) to occur at $\lambda = 4.5$ for equally sized domains; our model gives a value of $\lambda = 4.63$.

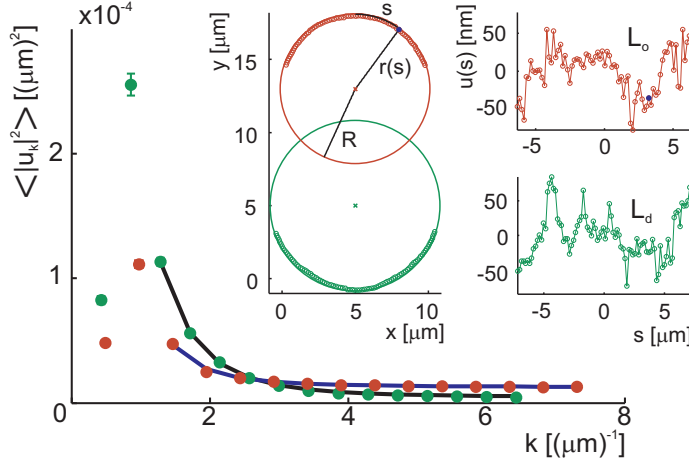
2.3 Experiment

Giant unilamellar vesicles (GUVs) were produced by electroformation from a mixture of 30 % DOPC, 50 % brain sphingomyelin, and 20 % cholesterol at 55 °C. Subsequently lowering the temperature to 20 °C resulted in the spontaneous formation of liquid ordered L_o and liquid disordered L_d domains on the vesicles. The L_d phase was stained by a small

amount of Rhodamine-DOPE (0.2 %). In order to stain the L_o phase a small amount (0.2 %) of the ganglioside GM1 was added, and subsequently cholera toxin labeled with Alexa 647 was bound to the GM1. The DOPC (1,2-di-oleoyl-sn-glycero-3-phosphocholine), sphingomyelin, cholesterol, Rhodamine-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl)), and GM1 were obtained from Avanti Polar Lipids; the Alexa labeled cholera toxin from Molecular Probes. For imaging we chose a wide-field epi-fluorescence setup (131) because short illumination times (1-5 ms) prevent shape fluctuations with short correlation times from being washed out. The raw data of a typical vesicle is shown in Fig. 2.1. The lateral resolution of the equatorial optical sections was limited by diffraction and pixelation effects. In the normal direction, however, a high (sub-pixel) accuracy was obtained. The upper inset in Fig. 2.1 shows a typical intensity profile along a line perpendicular to the contour. We determine numerically the profile's first derivative (lower inset in Fig. 2.1) and fit the central part around the maximum intensity with a straight line. The intercept with the x -axis gives the position of the vesicle edge. The positional accuracy achieved is typically 20 nm. The contours obtained were subsequently smoothed by a polynomial and all contours from the same vesicle (typically around 1000) were averaged to give the final result for the mean contour.

Spectra of the shape fluctuations were obtained from those parts of the contours that were nearly circular, *i.e.* far away from the neck domain. Fluctuations were determined for each single contour as the difference between the local radius r and the ensemble averaged radius R of a circle fitted to patches around the vesicles' poles: $u(s) = r(s) - R$ where s is the arclength along the circle, see Fig. 2.2.

The experimental fluctuation spectrum was obtained by Fourier transform as $u_k = \frac{1}{a} \int_{-a/2}^{a/2} ds r(s) e^{-ik \cdot s}$, where a is the arclength of the contour patch, and $k = n \cdot \frac{2\pi}{a}$ with n a non-zero integer. To derive a theoretical expression for the fluctuations spectrum we adopt the spectral analysis of a closed vesicle shell developed by Pécrciaux *et al.* (67). Additionally we have to take into account the finite patch size (83). As shown in (67) the fluctuation spectra of a sphere and a flat membrane differ only for the lowest modes. Consequently, we derive the spectrum of a flat membrane and omit the lowest modes in the analysis of the experiments. A flat membrane without overhangs or folds can be described as a height profile (Monge gauge) $h(\mathbf{r})$, where $h(\mathbf{r})$ is the height above the $z = 0$ plane at

**Figure 2.2**

Fluctuation spectra of the ordered (red circles) and disordered (green circles) domains. The corresponding best fits of Eq. (2.12) are shown in blue and black respectively. Inset: Typical real-space fluctuations along the vesicle perimeter.

position $\mathbf{r} = (x, y)$. Assuming periodic boundary conditions with spatial period L , we can use the following Fourier transformations

$$h(\mathbf{r}) = \sum_{\mathbf{q}} h_{\mathbf{q}} e^{i\mathbf{q}\cdot\mathbf{r}}, \quad (q_x, q_y) = \frac{2\pi}{L}(l_x, l_y), \quad (l_x, l_y) \in \mathbb{Z}^2$$

$$h_{\mathbf{q}} = \frac{1}{L^2} \int_{-L/2}^{L/2} dx \int_{-L/2}^{L/2} dy h(\mathbf{r}) e^{-i\mathbf{q}\cdot\mathbf{r}}$$

The Canham-Helfrich free energy for a flat membrane in Monge representation does not contain a gaussian curvature term due to periodic boundary conditions and absence of structures of higher topology ("handles"). The free energy therefore reads

$$H = \frac{1}{2} \int_{-L/2}^{L/2} dx \int_{-L/2}^{L/2} dy \left[\sigma (\nabla h(\mathbf{r}))^2 + \kappa (\nabla^2 h(\mathbf{r}))^2 \right]$$

$$= \frac{L^2}{2} \sum_{\mathbf{q}} [\sigma q^2 + \kappa q^4] |h_{\mathbf{q}}|^2$$

From the equipartition theorem it follows for the fluctuation spectrum

$$\langle |h_{\mathbf{q}}|^2 \rangle$$

$$\langle |h_{\mathbf{q}}|^2 \rangle = \frac{1}{L^2} \frac{k_B T}{\sigma q^2 + \kappa q^4}$$

Since we observe with the microscope an (optical) section of the membrane (the xz -plane), see Fig. 2.3, we cannot measure $h(\mathbf{r})$ but only $h(x, 0)$

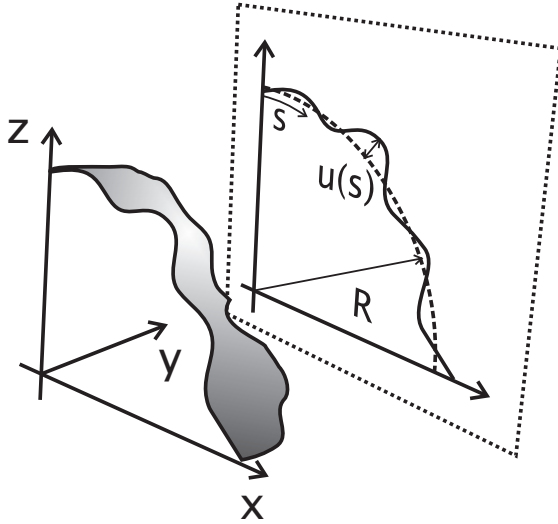


Figure 2.3

Optical section along the xz -plane. s is the contourlength, $u(s)$ is the deviation from the mean radius R at position s .

We define h_{q_x} as the Fourier components of the observable membrane profile $h(x, 0)$

$$\begin{aligned} h_{q_x} &:= \frac{1}{L} \int_{-L/2}^{L/2} dx \underbrace{h(x, 0)}_{\sum_{\mathbf{q}'} h_{\mathbf{q}'} e^{i q_x x}} e^{-i q_x x} \\ &= \sum_{\mathbf{q}'} h_{\mathbf{q}'} \underbrace{\frac{1}{L} \int_{-L/2}^{L/2} dx e^{i(q'_x - q_x)x}}_{\delta_{q_x, q'_x}} \\ &= \sum_{q'_y} h_{(q_x, q'_y)} \end{aligned}$$

If we take the ensemble mean we arrive at

$$\begin{aligned}
 \langle |h_{q_x}|^2 \rangle &= \sum_{q'_y, q''_y} \underbrace{\langle h_{(q_x, q'_y)} h_{(q_x, q''_y)}^* \rangle}_{\langle |h_{(q_x, q'_y)}|^2 \rangle \delta_{q'_y, q''_y}} = \sum_{q'_y} \langle |h_{(q_x, q'_y)}|^2 \rangle \\
 &\quad (\text{fluctuations with different } q_y \text{ are uncorrelated}) \\
 &= \frac{L}{2\pi} \int_{-\infty}^{\infty} dq'_y \langle |h_{(q_x, q'_y)}|^2 \rangle
 \end{aligned} \tag{2.7}$$

Integration by expansion into partial fractions results in

$$\begin{aligned}
 \langle |h_{q_x}|^2 \rangle &= \frac{k_B T}{2\pi L} \int_{-\infty}^{\infty} dq_y \frac{1}{(q_x^2 + q_y^2) \cdot ((\sigma + \kappa q_x^2) + \kappa q_y^2)} \\
 &= \frac{k_B T}{2\pi L \sigma} \left(\int_{-\infty}^{\infty} dq_y \frac{1}{(q_x^2 + q_y^2)} - \int_{-\infty}^{\infty} dq_y \frac{1}{((\frac{\sigma}{\kappa} + q_x^2) + q_y^2)} \right) \\
 &= \frac{k_B T}{2\pi L \sigma} \left(\frac{1}{q_x} \arctan\left(\frac{q_y}{q_x}\right) \Big|_{q_y \rightarrow -\infty}^{q_y \rightarrow \infty} \right. \\
 &\quad \left. - \frac{1}{\sqrt{\frac{\sigma}{\kappa} + q_x^2}} \arctan\left(\frac{q_y}{\frac{\sigma}{\kappa} + q_x^2}\right) \Big|_{q_y \rightarrow -\infty}^{q_y \rightarrow \infty} \right) \\
 \langle |h_{q_x}|^2 \rangle &= \frac{k_B T}{2L\sigma} \left(\frac{1}{q_x} - \frac{1}{\sqrt{\frac{\sigma}{\kappa} + q_x^2}} \right)
 \end{aligned} \tag{2.8}$$

The two transformations performed can be found in (168), p. 157 and p. 160 respectively. For tensionless membranes ($\sigma = 0$) or in the bending regime $q_x^2 \gg \frac{\sigma}{\kappa}$, the expression for the spectrum simplifies to

$$\langle |h_{q_x}|^2 \rangle \approx \frac{k_B T}{4L} \cdot \frac{1}{\kappa q_x^3} \tag{2.9}$$

The magnitude of short wavelength fluctuations only depends on the bending rigidity κ .

This model for the fluctuation spectrum of a flat membrane has to be adapted in two ways for the case of phase separated GUVs. We assume, as detailed above, that the vesicle is approximately spherical far away from the interface. In (67) it was shown that for higher modes the fluctuation spectrum of a flat membrane with periodicity $L = 2\pi R$ is (numerically) the same as that of a sphere with Radius R : For fluctuations with short wavelengths (= higher modes) it does not matter that the membrane is

curved on a length scale that is big compared to their wavelength. Therefore, we can in principle use the spectrum derived above, if we discard the lowest modes. However, the spherical part of the phase separated GUVs is not closed. Consequently, we have to derive the form of the spectrum for a finite membrane patch. Following (67) we choose $L = 2\pi R$ as the periodic interval and consider a patch of length a . For simplicity we choose a such that L is an integer multiple of a .

$$u_k := \frac{1}{a} \int_{-a/2}^{a/2} ds u(s) e^{-ik \cdot s} \quad , \quad u(s) := h(s, 0) - R \quad , \quad a = \frac{L}{m} \quad , \quad m \in \mathbb{Z}$$

$$u(s) = \sum_k u_k e^{ik \cdot s} \quad , \quad k = n \cdot \frac{2\pi}{a} = n \cdot m \cdot \frac{2\pi}{L} \quad , \quad n \in \mathbb{Z}$$

where R is the mean radius of a fitted circle and $u(s)$ is the radial distance between this circle and the measured contour, s is the arclength along the fitted circle and $L = 2\pi R$, see Fig. 2.3.

Following ideas developed in (83) we calculate the fluctuation spectrum for a patch in terms of the full spectrum

$$\begin{aligned} \langle |u_k|^2 \rangle &= \frac{1}{a^2} \int_{-a/2}^{a/2} ds \int_{-a/2}^{a/2} ds' \langle u(s) u(s') \rangle e^{ik \cdot (s-s')} \\ &= \frac{1}{a^2} \sum_{q_x, q'_x} \int_{-a/2}^{a/2} ds \int_{-a/2}^{a/2} ds' \underbrace{\langle u_{q_x} u_{q'_x} \rangle e^{iq_x \cdot s}}_{\rightarrow -\langle u_{q_x}^* u_{q'_x} \rangle e^{-iq_x \cdot s}} e^{iq'_x \cdot s'} e^{ik \cdot (s-s')} \\ &= \sum_{q_x} \frac{k_B T}{2L\sigma} \left(\frac{1}{q_x} - \frac{1}{\sqrt{\frac{\sigma}{\kappa} + q_x^2}} \right) \underbrace{\left(\frac{\sin((k - q_x) \frac{a}{2})}{(k - q_x) \frac{a}{2}} \right)^2}_{\rightarrow \delta_{k, q_x} \text{ for } a \rightarrow L} \end{aligned} \quad (2.10)$$

For $a = L$ we recover the fluctuation spectrum of a closed sphere.

An experimental detail, which further complicates the calculation of the fluctuation spectrum, is that membrane contours are averaged over the camera integration time t (= illumination time). Consequently, we observe time averaged fluctuations:

$$\overline{u(s)} = \frac{1}{t} \int_0^t u(s, t') dt'$$

To determine the influence of time averaging on the spectrum we need to know the correlation times of the fluctuation modes (67, 169)

$$\langle h_{\mathbf{q}}^*(t_1) h_{\mathbf{q}'}(t_2) \rangle = \delta_{\mathbf{q}, \mathbf{q}'} \langle |h_{\mathbf{q}}|^2 \rangle \exp\left(-\frac{|t_1 - t_2|}{\tau_q}\right) \text{ with } \tau_q = \frac{4\eta q}{\sigma q^2 + \kappa q^4}$$

where τ_q is the correlation time and η is the bulk viscosity of the medium surrounding the membrane.

The time averaged spectrum is then

$$\begin{aligned} \overline{\langle h_{\mathbf{q}}^* h_{\mathbf{q}'} \rangle} &= \frac{1}{t^2} \int_0^t dt_1 \int_0^t dt_2 \langle h_{\mathbf{q}}^*(t_1) h_{\mathbf{q}'}(t_2) \rangle \\ &= \delta_{\mathbf{q}, \mathbf{q}'} \langle |h_{\mathbf{q}}|^2 \rangle 2 \frac{\tau_q^2}{t^2} \left(\frac{t}{\tau_q} + \exp(-t/\tau_q) - 1 \right) \\ &= \delta_{\mathbf{q}, \mathbf{q}'} \frac{k_B T}{L^2 2\eta q} \tau_q \frac{\tau_q^2}{t^2} \left(\frac{t}{\tau_q} + \exp(-t/\tau_q) - 1 \right) \end{aligned} \quad (2.11)$$

Combination of equations (2.7), (2.8), (2.10), (2.11) gives the final result for the time averaged fluctuation spectrum of a membrane patch

$$\begin{aligned} \overline{\langle |u_k|^2 \rangle} &= \sum_{q_x} \frac{L}{2\pi} \int_{-\infty}^{\infty} dq'_y \overline{\langle |h_{(q_x, q'_y)}|^2 \rangle} \left(\frac{\sin((k - q_x) \frac{a}{2})}{(k - q_x) \frac{a}{2}} \right)^2 \\ &= \sum_{q_x} \left(\frac{\sin((k - q_x) \frac{a}{2})}{(k - q_x) \frac{a}{2}} \right)^2 \\ &\quad \times \frac{L}{2\pi} \int_{-\infty}^{\infty} dq_y \frac{k_B T}{L^2 2\eta q} \tau_q \frac{\tau_q^2}{t^2} \left(\frac{t}{\tau_q} + \exp(-t/\tau_q) - 1 \right) \\ &\text{with } q = |\mathbf{q}|, \quad \mathbf{q} = (q_x, q_y) \end{aligned} \quad (2.12)$$

We fit this expression to the experimentally obtained spectra with σ and κ as independent fit parameters. Separate fits are performed for the two phases. Examples of such fits are shown in Fig. 2.2. The two lowest modes are omitted since we derived the fluctuation spectrum for a flat membrane.

Theoretically, the spectrum should decay to 0 in the short wavelength limit. However, due to experimental limitations, there is a constant offset: Even if the membrane is *fixed*, we would observe fluctuations due to the finite positional accuracy ε for determination of the membrane contour. For a *fixed* membrane the probability density to observe an excursion of

size u is given by a Gaussian with standard deviation ε

$$f(u) = \frac{1}{\varepsilon\sqrt{2\pi}} \exp\left(-\frac{u^2}{2\varepsilon^2}\right)$$

Since real shape fluctuations and apparent fluctuations due to the finite positional accuracy are uncorrelated we can write

$$\langle |u_k|^2 \rangle_{\text{meas}} = \langle |u_k|^2 \rangle_{\text{real}} + \frac{1}{a^2} \int_{-a/2}^{a/2} ds \int_{-a/2}^{a/2} ds' \langle u(s)u(s') \rangle e^{ik \cdot (s-s')}$$

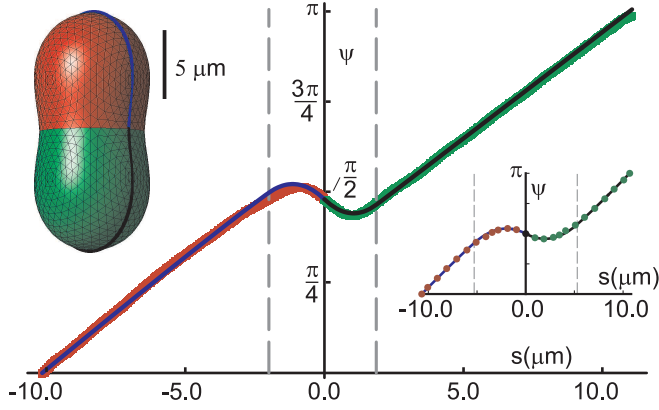
If we determine the membrane contour at N points, we get

$$\begin{aligned} \langle |u_k|^2 \rangle_{\text{meas}} &= \langle |u_k|^2 \rangle_{\text{real}} + \frac{1}{N^2} \sum_{i,j=1}^N \underbrace{\langle u(s_i)u(s_j) \rangle}_{\delta_{i,j} \langle u^2 \rangle} e^{ik \cdot (s_i - s_j)} \\ &= \langle |u_k|^2 \rangle_{\text{real}} + \frac{\varepsilon^2}{N} \end{aligned}$$

where $\langle |u_k|^2 \rangle_{\text{meas}}$ is the measured fluctuation spectrum and $\langle |u_k|^2 \rangle_{\text{real}}$ is the real spectrum. The positional accuracy ε can then be determined from the offset ε^2/N .

2.4 Results

Fits of the fluctuation spectra using Eq. (2.12) give the values of the bending moduli and surface tensions of the two phases. Using these values, we fit the experimentally obtained vesicle shapes with the model described above. This leaves us with two parameters: the line tension τ between the two phases and the difference $\Delta\kappa_G$ between their Gaussian moduli. Since the experimental data show that ψ at the domain boundary follows a straight, continuous line we further assume that the derivative $\dot{\psi}$ is continuous at the domain boundary (as suggested before (91, 166)). Imposing this additional condition fixes the value of $\Delta\kappa_G$ for given τ , leaving us with a single free parameter to fully describe the system. A two-parameter fit without the continuity condition on $\dot{\psi}$ at the domain boundary gives the same results within the experimental accuracy. By fitting the experimental data, we directly extract the line tension. An example fit is shown in Fig. 2.4.

**Figure 2.4**

Example for an experimentally obtained $\psi(s)$ plot (red: L_o phase, green: L_d phase) together with the best fit of the model (blue: L_o phase, black: L_d phase). The dashed lines mark the transition points between the neck and bulk domains. Insets: Fit to a numerically obtained shape (using Surface Evolver).

Values found for the bending moduli are $8 \pm 1 \cdot 10^{-19}$ J for the L_o domain and $1.9 \pm 0.5 \cdot 10^{-19}$ J for the L_d domain. For the line tension we found a value of 1.2 ± 0.3 pN, which is in the same range as that estimated by Baumgart *et al.* (70). Finally, the difference in Gaussian moduli is about $3 \pm 1 \cdot 10^{-19}$ J, in accordance with the earlier established upper bound ($\kappa_G \leq -0.83\kappa$) reported by Siegel and Kozlov (160). An overview of the results is given in Table 2.1.

	σ_d ($10^{-7} \frac{N}{m}$)	κ_d (10^{-19} J)	σ_o ($10^{-7} \frac{N}{m}$)	κ_o (10^{-19} J)	τ (pN)	$\Delta\kappa_G$ (10^{-19} J)
1	2.8 ± 0.2	2.2 ± 0.1	0.3 ± 0.3	8.0 ± 1.3	1.5 ± 0.3	2.5 ± 2
2	5.8 ± 0.5	1.8 ± 0.2	2.1 ± 0.4	8.2 ± 1.5	1.2 ± 0.4	2.0 ± 2
3	3.5 ± 0.3	2.0 ± 0.1	2.0 ± 0.5	8.2 ± 1.4	1.2 ± 0.3	2.5 ± 2
4	2.8 ± 0.2	1.9 ± 0.1	2.5 ± 0.5	8.3 ± 1.2	1.2 ± 0.4	4.0 ± 2
5	2.3 ± 0.1	1.6 ± 0.1	0.6 ± 0.3	8.0 ± 1.6	1.1 ± 0.5	4.0 ± 3

Table 2.1

Values of the material parameters for five different vesicles. The surface tensions and bending moduli of the L_d and L_o phase are determined from the fluctuation spectrum; the line tension and difference in Gaussian moduli are subsequently determined using our analytical model.

2.5 Discussion

Ultimately, one worries about the membrane's elastic parameters because their precise magnitude has important consequences for the morphology and dynamics of cells. The literature is replete with theoretical speculations which depend strongly on, among others, the line tension. While the values we report apply to reconstituted vesicles, we can nonetheless use them in some of these models to explore possible implications for cellular membranes. The majority of the investigated vesicles finally evolved into the fully phase separated state. This finding is in agreement with previous work by Frolov *et al.* (159), which predicts, for line tensions larger than 0.4 pN, complete phase separation for systems in equilibrium. It should be noted that the line tension found is also smaller than the critical line tension leading to budding: recent results by Liu *et al.* (85) show that for endocytosis by means of membrane budding both high line tensions (> 10 pN) and large domains are necessary. Therefore nanodomains will be stable and will not bud. In cells, however, additional mechanisms must be considered. To explain the absence of large domains *in vivo*, Turner *et al.* (86) make use of a continuous membrane recycling mechanism. For the membrane parameters we have determined such a mechanism predicts asymptotic domains of ~ 10 nm in diameter. Our results, in combination with active membrane recycling therefore support a minimal physical mechanism as a stabilizer for nanodomains in cells. A separate effect, purely based on the elastic properties of membranes may further stabilize smaller domains *in vivo*. Recently, Yanagisawa *et al.* explored the consequences of a repulsive interaction between nearby buds (88) and reported that such interactions can arrest the phase separation kinetics. The elastic perturbations induced by domains in the membrane, as described in this Letter, are obvious candidates for producing additional interactions between buds at any distance, further assisting in the creation of such a kinetic arrest. As Müller *et al.* have shown for a flat membrane, two distortions on the same side of an infinite flat membrane repel on all length scales (55). The experimental observation of multiple domains ordered in (quasi-)crystalline fashion in model membranes (70) strongly suggests a similar repulsive interaction in spherical vesicle systems. This is indeed evidenced by preliminary numerical exploration of this system using Surface Evolver (167). It is of course straightforward to adapt the scheme outlined above to include long-range interactions between transmembrane proteins that impose a curvature on the membrane, *e.g.* scaffolding pro-

teins (42, 43, 53). Membrane mediated interactions act over length scales much larger than Van der Waals or electrostatic interactions and could provide an alternative or additional physical mechanism for processes like protein clustering and domain formation. Our results and methods allow not only to determine the parameters relevant to processes like these, but also give a practical analytical handle on the shapes involved. This, in turn, will help decide between competing proposals for mechanisms involving membrane bending: protein interactions, endocytosis and the formation and stabilization of functional membrane domains.

CHAPTER 3

MEMBRANE MEDIATED INTERACTIONS MEASURED USING MEMBRANE DOMAINS¹

Cell membrane organization is the result of the collective effect of many driving forces. Several of these, such as electrostatic and van der Waals forces, have been identified and studied in detail. Here we investigate and quantify another force, the interaction between inclusions via deformations of the membrane shape. For electrically neutral systems this interaction is the dominant organizing force. As a model system to study membrane mediated interactions we use phase separated biomimetic vesicles which exhibit coexistence of liquid ordered and liquid disordered lipid domains. The membrane mediated interactions between these domains lead to a rich variety of effects, including the creation of long range order and the setting of a preferred domain size. Our findings also apply to the interaction of membrane protein patches which induce similar membrane shape deformations and hence experience similar interactions.

¹This chapter is based on: S. Semrau, T. Idema, C. Storm, T. Schmidt, Membrane-mediated interactions measured using membrane domains, *Biophys. J.*, **96** (12), 4906-4915, (2009)

3.1 Introduction

Lipid bilayer membranes enclose and compartmentalize the living cell, and as such represent the single most important barrier that cellular sensing and transport processes face (170). The detection of, and adequate response to, extracellular cues in particular is strongly bound to the membrane. Rather than allow ligands to pass through the membrane, changes in external concentrations of specific agonists are typically registered by transmembrane proteins and protein complexes. The spatial organization of such proteins is crucial to the successful transduction of signals across the membrane, and facilitates many cellular processes (45, 170). This organization within the membrane has been the subject of intense studies, and represents a fundamental biological challenge: how is it that supramolecular organization comes about and persists in the two-dimensional fluid environment of the membrane? After all, in a perfectly liquid environment diffusion would tend to strongly counteract pattern formation and would quickly erase any significant density gradients. Moreover, traditionally considered protein-protein interactions (hydrophilic/hydrophobic, electrostatic, van der Waals) tend to be either too short-ranged or too weak to effectively drive the formation of heterogeneities.

Protein interactions mediated by the membrane have been suggested as a possible mechanism to overcome the limitations set by short-ranged conventional interactions. The membrane may effectively mediate protein interactions in several ways. The first is by creating local inhomogeneities in membrane composition, particularly, the emergence of small domains enriched in particular lipid species (8, 150). These domains may present transient or persistent target sites for protein aggregation due to protein confinement or specific lipid-protein interactions. A second possibility is that single lipids or proteins interact via hydrophobic mismatches. If the length of the hydrophobic domain of a protein or lipid does not match the thickness of the surrounding membrane, this configuration will carry an energy penalty. To reduce that penalty, lipids or proteins may aggregate with similar-sized ones. The interaction due to hydrophobic mismatch is short-ranged and independent of overall membrane curvature (171). A final possibility is that proteins locally distort the membrane shape (53–55, 172). Such distortions lead to an effective interaction between them through the differential curvature they impart. Especially aggregates of proteins could interact via membrane curvature. Such interactions would

have biological implications, for example, for the assembly of protein coats and endocytosis (45).

Here we study the existence and magnitude of the last type of membrane mediated interactions. We do so by considering the dynamics of domains on partially phase separated vesicles containing cholesterol and two other species of lipids (60). While no proteins are present in our system, these small lipid domains mimic the proposed behavior of proteins (70). They, too, locally distort the shape of the membrane. Working with domains carries two great advantages over using actual proteins. Firstly, the domains interact only through the membrane shape deformations they induce. Secondly, they are straightforward to visualize and track. Earlier studies of the same system by Yanagisawa et al. (88) focused on the dynamics of domain growth. They described a slowing down of domain coalescence due to membrane mediated interactions. Rozovsky et al. (87) reported the formation of regular patterns in a similar system. In their experiments the shape of the vesicle was strongly coupled to phase separation due to substrate adhesion. In this study we use domains on freely suspended giant vesicles as a probe to demonstrate the existence of membrane mediated interactions. We develop a theoretical model that predicts the existence of partially budded domains in this system which is a prerequisite for membrane mediated interactions. We measure the distribution of domain sizes and find a pronounced preferred length scale. By analysis of the fluctuations of domain positions we quantify the strength of membrane interactions and find a nontrivial dependence of the interaction strength on domain size. Those effects are captured qualitatively in a simple model. Our findings shed new light on intramembrane interactions between protein patches. Moreover, they also yield new information on the domain size distribution and the stability of the widely reported microphase separation in multicomponent biomimetic membranes.

3.2 Materials and Methods

3.2.1 GUV formation

Giant unilamellar vesicles (GUVs) were produced by electroformation in a flow chamber (62, 64) from a mixture of 30% DOPC, 50% brain sphingomyelin, and 20% cholesterol at 55°C. The liquid disordered L_d phase was stained by a small amount of Rhodamine-DOPE (0.2%-0.4%), the liquid ordered L_o with a small amount (0.2%-0.4%) of perylene. The

DOPC (1,2-di-oleoyl-sn-glycero-3-phosphocholine), sphingomyelin, cholesterol and Rhodamine-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl)) were obtained from Avanti Polar Lipids (Alabaster, AL, USA); the perylene from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands).

After formation of the GUVs the buffer (256 mM sucrose) was partially exchanged by a buffer with a higher osmolarity (335 mM sucrose) resulting in a difference of osmolarity of about 40-50 mM between inside and outside of the vesicles. Subsequently lowering the temperature to 20°C resulted in the spontaneous nucleation of liquid ordered L_o and liquid disordered L_d domains on the vesicles. All reported observations were made on vesicles that show partially budded domains which are stable over extended periods of time. In total, 21 vesicles were recorded.

Vesicles were imaged at video rate with a Watec 902H2 supreme CCD camera attached to an inverted microscope (Axiovert 40 CFL, Zeiss). The sample was illuminated continuously by a mercury lamp (HBO 50, Zeiss) and suitable excitation filters. Fluorescence signal was collected using appropriate dichroic mirrors and emission filters.

3.2.2 Image analysis

First, an equatorial image of the vesicle is taken to determine its radius and center position, see Fig.3.1.

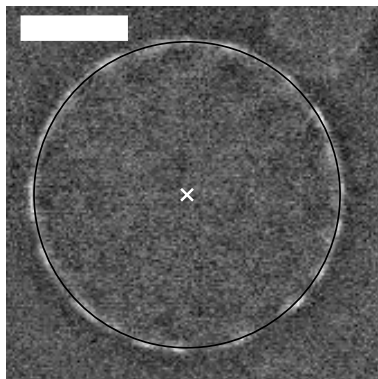


Figure 3.1

L_d phase of a phase separated vesicle with fitted circle (black) superimposed. The cross marks the position of the center. Scalebar: 20 μm .

After taking the equatorial image the focus is moved to the top of the vesicle and the movement of domains is followed for several minutes. Every frame of those movies (Fig. 3.2c) is treated with a band pass Fourier

filter to eliminate noise and background and a region of interest around the center of the vesicle is chosen (Fig. 3.2d). The filtered grayscale image is subsequently transformed to a binary image by thresholding (Fig. 3.2e). The positions of the domains are determined from the centroids of the domains (i.e., the center of mass, where mass corresponds to pixel intensity here). The short and long axes of the domains are calculated from the moment of inertia tensor (Fig. 3.2f). Since the domains are in a liquid phase their boundaries are circular. They appear elliptical due to the projection onto a plane. The real radius of a domain is given by the long axis of the observed ellipse.

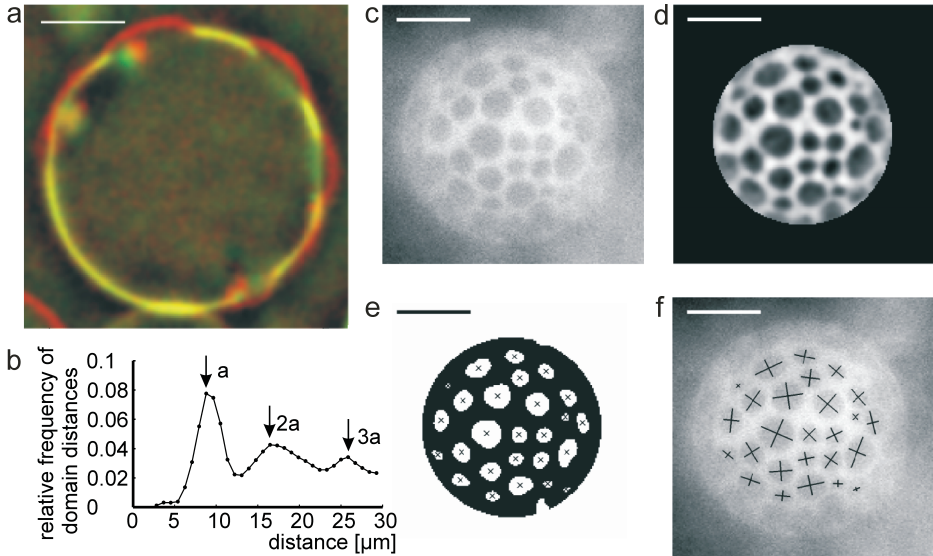


Figure 3.2

Analysis of experimental data. (a) Cross-section of a partially budded vesicle. Overlay of 405 nm excitation (perylene, red / dark gray) and 546 nm excitation (rhodamine, yellow / light gray). (b) Typical radial distribution function for the center-center distances of the domains on a single vesicle. The nearest neighbor distance is denoted by a . (c-f) Image analysis. The L_d phase is stained and appears bright in the images; the L_o phase appears dark. (c) Raw image; (d) filtered image with region of interest on top of the vesicle; (e) filtered image converted to binary image by thresholding, the crosses mark the centroids of the domains; (f) raw image with long and short axes of the domains overlaid. All scalebars: 20 μm.

The vesicle is assumed to be approximately spherical. Hence the z position of the domains relative to the equatorial plane can be calculated

from the position of the centroids and the center of the vesicle. All domain radii and distances between domains are measured along the vesicle surface.

3.3 Evidence for interactions

We experimentally studied the dynamics of tricomponent GUVs. Under appropriate conditions on composition and temperature, the lipids in such vesicles phase separate into liquid ordered (L_o) and liquid disordered (L_d) domains (73). In our system we typically observe many L_o domains in a L_d background, see Fig. 3.2. After preparation by means of electroformation the vesicles have a spherical shape. By increasing the osmotic pressure outside the vesicle we produce a slight increase in surface to volume ratio. For this reason some of the vesicles show partially budded L_o domains, see Fig. 3.2a. Those domains possess long term stability (in experiments we observed stability on the time scale of several hours). In contrast, ‘flat’ domains, which have the same curvature as the vesicle as a whole, rapidly fuse until complete phase separation is attained (20, 88) (Chap. 2 of this thesis).

The stability of the vesicles with budded domains indicates that the domains experience a repulsive interaction that prevents them from fusing. This interaction also affects the distribution of domain distances (radial distribution function) and domain sizes.

3.3.1 Radial distribution function

Fig. 3.2b shows the radial distribution function (rdf) of the center-to-center distance of domains for a typical vesicle. The first (and highest) maximum in the rdf corresponds to the first coordination shell, i.e., the nearest neighbors. The distance between nearest neighbors is denoted by a . On average $a = 9 \mu\text{m}$, while the radius of a domain is on average $3 \mu\text{m}$ and the vesicle radius equals $34 \mu\text{m}$ on average. Fig. 3.2b clearly shows two additional maxima roughly at $2a$ and $3a$ which correspond to the second and third coordination shell. The rdf therefore indicates that the domains are not randomly distributed but that instead their positions are correlated. Consequently the system of diffusing domains can be characterized as a two-dimensional liquid with interactions. Since a exceeds the typical domain radius by a factor of 3, this interaction is different from mere hard core repulsion between the domains.

3.3.2 Size distribution

Fig. 3.3 shows the domain size distribution of all observed vesicles. The distribution is not uniform, but instead shows an absolute maximum, corresponding to a preferred domain size. Moreover, there is a long tail to larger domain sizes which drops off exponentially, as can be seen in a logscale plot (Fig. 3.3 inset). This nonuniform distribution can be understood in a picture that includes both domain fusions and domain interactions.

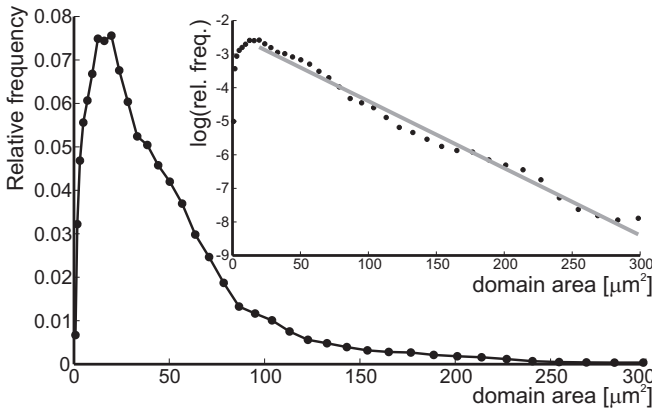


Figure 3.3

Distribution of domain sizes on all 24 vesicles. Inset: logarithmic plot of the domain size distribution shows an exponential decay towards large domains (solid line).

As was already observed by Yanagisawa et al. (88) we find that domains fuse when they are small. However, due to the repulsive interaction the fusion of domains becomes kinetically hindered and slows down significantly with increasing domain size. When the repulsive interaction has grown to the size of the thermal energy ($k_B T$), the fusion process will come to a halt and the vesicle with multiple domains enters the metastable ‘kinetically arrested’ state which we observe in the experiments. Due to the finite available amount of domain area the frequency of size occurrence decays exponentially for large domains (Fig. 3.3).

To check if the local maximum and the exponential tail observed in the experimental domain size distribution can be explained by mutual repulsion of domains, we performed Monte Carlo simulations of domain coalescence. We assume that the rate for the fusion of two domains of areas n and m can be written as the product of two factors: the rate for random encounter by diffusion $k_{\text{diff}}(\{N_n\})$, which may depend on the distribution of domain sizes $\{N_n\}$, and the probability $p_{n,m}^{\text{merge}}$ for domain

merger if the domains are close to each other:

$$k_{n,m} = p_{n,m}^{\text{merge}} k_{\text{diff}}(\{N_n\}). \quad (3.1)$$

Our simulations start with $1/\varepsilon$ domains of identical size, where ε is defined as the initial domain area. During the simulation the domains are fused randomly with the rates given above. The fusion rate is converted to a fusion probability $p_{n,m}$ by multiplication with a small time step Δt . Since there are $\frac{1}{2}N(N-1)$ possible pairings of N domains we write the fusion probability as:

$$p_{n,m} = k_{n,m}\Delta t = \frac{1}{\frac{1}{2}N(N-1)} p_{n,m}^{\text{merge}} \left(\frac{1}{2}N(N-1) \right) k_{\text{diff}}(\{N_n\})\Delta t, \quad (3.2)$$

with the total number of domains given by $N = \sum_n N_n$.

If the time step Δt is chosen to be $\Delta t = [(\frac{1}{2}N(N-1))k_{\text{diff}}(\{N_n\})]^{-1}$ the fusion probability becomes $p_{n,m} = \frac{1}{\frac{1}{2}N(N-1)} p_{n,m}^{\text{merge}}$.

In the following we briefly sketch the Monte Carlo algorithm we used, details can be found in (173). In each Monte Carlo step, first a pair of domains is chosen randomly (which corresponds to the factor $1/(\frac{1}{2}N(N-1))$ in $p_{n,m}$) and the Monte Carlo time is increased by Δt . With a probability of $p_{n,m}^{\text{merge}}$ the domain fusion is executed. In agreement with our experimental observations we do not allow for scission events, i.e. the fission of a domain into two smaller domains. Due to the high line tension such events never occur in our experiments.

To show that this Monte Carlo scheme results in the correct domain fusion dynamics, first, we consider a model for which an analytical solution can be found and compare this result to the outcome of the simulations. For short enough times, when the system is still far from complete phase separation, the evolution of domain sizes can be modeled by a master equation (86):

$$\dot{N}_n = \frac{1}{2} \sum_{m=1}^{n-1} k_{m,n-m} N_m N_{n-m} - \sum_{m=1}^{\infty} k_{n,m} N_n N_m \quad (3.3)$$

where N_n is the number of domains with area n , $k_{n,m}$ the fusion rate for domains of area n and m and the dot refers to the time derivative. A master equation approach disregards the discrete nature of domain numbers

and is therefore only applicable for domain numbers much bigger than 1, i.e. a long time before complete phase separation.

If we neglect the possibility of scission and the fusion rate is assumed to be independent of domain area ($k_{n,m} = k$) the continuous master equation

$$\frac{\partial}{\partial t} N(x, t) = k \left(\frac{1}{2} \int_0^x dy N(y, t) N(x - y, t) - \int_0^\infty dy N(x, t) N(y, t) \right) \quad (3.4)$$

can be solved exactly. The ansatz $N(x, t) = a(t) \exp(-b(t)x)$ results in a system of ordinary differential equations for the functions a and b :

$$\begin{cases} \dot{a}(t) = -\frac{k}{2} a(t) \\ \dot{b}(t) = -k \frac{a^2(t)}{b(t)} \end{cases} \Rightarrow \begin{cases} a(t) = (k_0 + \frac{k}{2}t)^{-2} \\ b(t) = (k_0 + \frac{k}{2}t)^{-1} \end{cases} \quad (3.5)$$

With the initial condition of $1/\varepsilon$ equally sized domains of area ε and $\varepsilon \rightarrow 0$ the solution is:

$$N(x, t) = \frac{1}{\left(\frac{k}{2}t\right)^2} e^{-\frac{1}{\frac{k}{2}t}x} \quad (3.6)$$

While the total domain area is constant

$$\int_0^\infty dx x N(x, t) = 1, \quad (3.7)$$

the total number of domains decays over time

$$\int_0^\infty dx N(x, t) = \frac{1}{\frac{k}{2}t}, \quad (3.8)$$

and consequently the average domain size increases

$$\frac{\int_0^\infty dx x N(x, t)}{\int_0^\infty dx N(x, t)} = \frac{k}{2}t. \quad (3.9)$$

Obviously, the master equation approach is only valid for $t \ll \frac{2}{k}$.

The solution of the continuous master equation is an approximate solution of the discrete master equation if the distribution is binned with binsize ε

$$N_n(t) \approx \varepsilon \frac{1}{\left(\frac{k}{2}t\right)^2} e^{-\frac{1}{\frac{k}{2}t}n} \quad (3.10)$$

The solution is an exponential distribution for all times, which suggests that the exponential tail observed in experiments is simply a consequence of conserved total domain area.

Figure 3.4a shows a comparison of the exact solution Eq. 3.10 of the continuous master equation to Monte Carlo simulations results for the corresponding discrete master equation. The good agreement proves the validity of our Monte Carlo scheme.

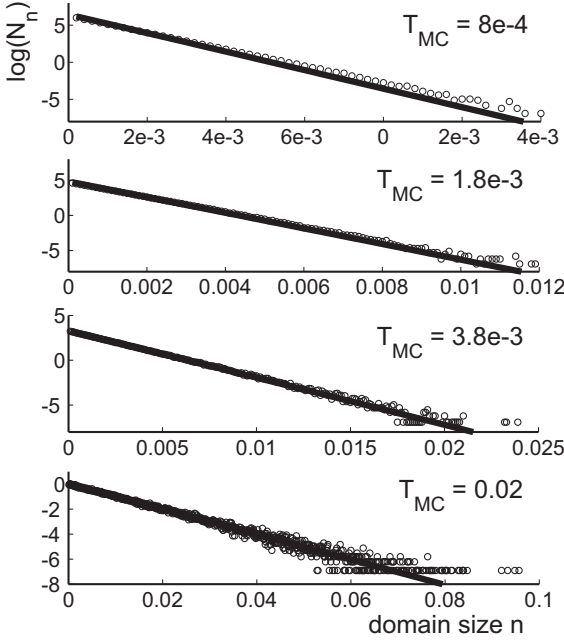


Figure 3.4

Domain size distributions determined using Monte Carlo simulations. Domain size distribution for 4 different Monte Carlo times averaged over 1000 simulation runs (open circles) with theoretically expected distribution (solid line) for fusion rate $k = 1$. Initial condition: 10^4 domains of area $\varepsilon = 10^{-4}$.

In order to take the spatial distribution and diffusion of domains into consideration we adopt the scaling argument used in (88) and (86): The time τ_{diff} for two domains to encounter each other at random due to diffusion scales like $\tau_{\text{diff}} \propto \langle d^2 \rangle / D(d)$ with d the domain radius and $D(d)$ the diffusion constant. Since we observe only a weak dependence of the diffusion coefficient on domain size ($D(d) \approx D$, see Fig. S4), we set $k_{\text{diff}}(\{N_n\}) = \pi / \langle A \rangle$ with the average domain area $\langle A \rangle = \frac{1}{N} \sum_n n N_n$. This rate should give the correct time scale for domain fusion apart from a constant prefactor. To gauge the simulations with real experimental time scales, we let the system evolve to complete phase separation for non-interacting domains ($p_{n,m}^{\text{merge}} = 1$) and compare the resulting Monte Carlo time to measured time scales. In the case of (flat) domains, which

are free to fuse, the time needed for complete separation was determined experimentally (see (88), normal coarsening) and is about 1-10 minutes. The corresponding Monte Carlo time in our simulations is $T_{MC} \approx 2$. Fig. 3.5a shows intermediate domain size distributions for four different Monte Carlo times. Clearly, the exponential behavior is conserved in the presence of diffusion and the typical lengthscale of that distribution (i.e., domain size) increases over time.

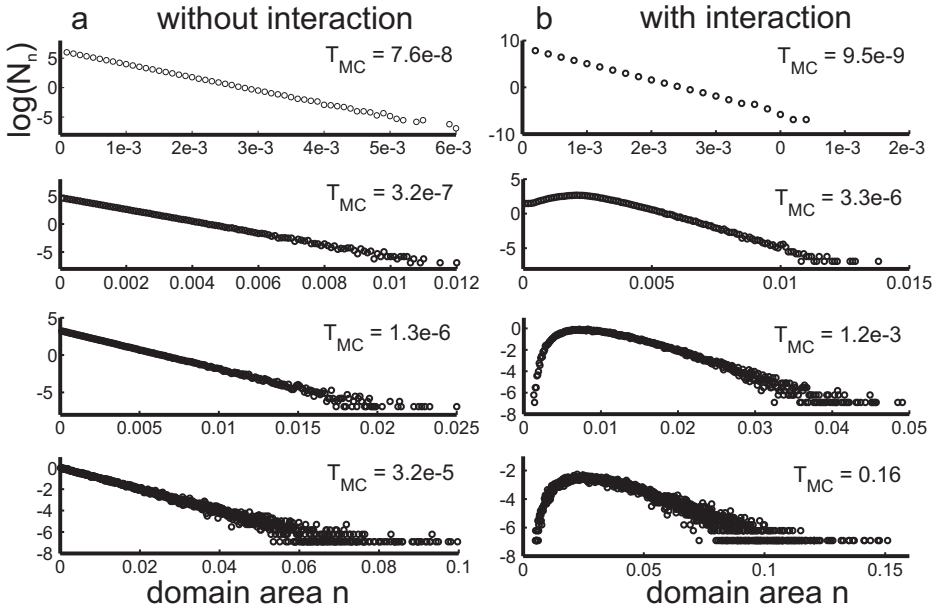


Figure 3.5

Domain size distributions determined using Monte Carlo simulations. (a) Distribution for four different Monte Carlo times averaged over 1000 simulation runs (open circles) including diffusion. Initial condition: 10^4 domains of area $\varepsilon = 10^{-4}$. (b) Distribution for four different Monte Carlo times averaged over 1000 simulation runs (open circles) including diffusion and interaction of domains. Here $p_{n,m}^{\text{merge}} = 10^{-6}/(n * m)$. Initial condition: 10^4 domains of area $\varepsilon = 10^{-4}$.

In the kinetic hindrance model for budded domains the probability for merger of two neighboring domains decreases with domain size. Hence we assume $p_{n,m}^{\text{merge}} = c/(n*m)$. (Since we do not attempt to obtain quantitative agreement with the experimental results, any probability that decreases monotonically with domain sizes would be acceptable as well.). Fig. 3.5b shows intermediate domain size distributions for 4 different Monte Carlo

times. The simulations reproduce the two qualitative features observed in experiments: the local maximum and the exponential tail, see Fig. 3.3. We find that at $T_{\text{MC}} \approx 175$ phase separation is still not complete. This is much longer than the time we found for complete phase separation in the case without interactions ($T_{\text{MC}} \approx 2$). The Monte Carlo simulations therefore show that incomplete phase separation is a quasistatic state.

3.4 Domain budding

The experimentally observed distributions of domain distances and sizes can be explained by a repulsive membrane mediated interaction between the domains. Domains that partially ‘bud out’ from the vesicle locally deform the membrane around them. Placing two budded domains close together causes this deformation to be larger, carrying a larger energy and resulting in an effective force between them. This membrane mediated force is therefore a direct consequence of the fact that the domains *partially* bud out from the vesicle. In this section we analyze the energetics of this partial budding process.

The first systematic study of domain budding was performed by Lipowsky (90). He modeled the domains as either circular disks in, or spherical caps on, a flat background. Domain budding is then a consequence of a tradeoff between two competing forces, which we will treat here in a coarse-grained, mean-field manner. For a more detailed view on the microscopic processes involved we refer to reviews by Lipowsky et al. (174) and Seifert (2). The first force is the line tension between the L_o domain and the L_d background, which favors budding because it reduces the length of the domain boundary. On the other hand the bending energy of the L_o domain resists budding because a budded domain has a higher curvature. Lipowsky found that there is a critical domain size at which there is a transition between an unbudded (or ‘flat’) state and a fully budded domain. This lengthscale is called the *invagination length*, given by $\xi = \kappa_o/\tau$, with κ_o the bending modulus of the L_o phase and τ the line tension on the domain boundary; in our experimental vesicles we have $\kappa_o \sim 8.0 \cdot 10^{-19}\text{J}$ and $\tau \sim 1.2\text{pN}$, giving $\xi \sim 0.7\mu\text{m}$ (20) (Chap. 2 of this thesis). The invagination length therefore sets the length scale at which we expect to find the first occurrence of domain budding. Although we occasionally see domains splitting off from the vesicle completely, we mostly observe partially budded domains. In the model proposed by Lipowsky

partial budding is not possible, suggesting that we need to consider additional constraints on, for example, the vesicle area and volume, and/or additional energy contributions. Such constraints were also studied by Jülicher and Lipowsky (91, 166). They used numerical methods to find the minimal-energy shape of a L_d vesicle with a single L_o domain. Their results confirm the finding by Lipowsky that there is a critical domain size for budding. Moreover, they found that a constraint on the volume of the vesicle only changes the budding point but does not modify the qualitative budding behavior. In the following we show that it is not sufficient to just include area and volume constraints to explain the shape of our experimental vesicles. If we also allow for stretching of the membrane, we do get the partially budded vesicle shapes.

In general, the equilibrium shape of the membrane of a GUV is found by minimizing the associated ‘shape energy’ functional under appropriate constraints on the total membrane area and enclosed volume. The functional is composed of several contributions, reflecting the energy associated with the deformation of the membrane and the effect of phase separation of the different lipids into domains. The contribution due to bending of the membrane (the ‘bending energy’) is given by the Canham-Helfrich energy functional (81, 82):

$$\mathcal{E}_{CH} = \mathcal{E}_{\text{curv}} + \mathcal{E}_{\text{Gauss}} = \int \left[\frac{\kappa}{2} (2H)^2 + \kappa_G K \right] dS. \quad (3.11)$$

Here H and K are the mean and Gaussian curvature of the membrane respectively, and κ and κ_G the bending and Gaussian moduli. We have not included a spontaneous curvature, because in our experimental system the membrane has ample time to relax any asymmetries between the membrane leaflets. Using the Gauss-Bonnet theorem, we find that the integral over the Gaussian curvature over a continuous patch of membrane, such as one of our L_o domains or the L_d background, yields a constant bulk contribution (which we can disregard) plus a boundary term (175). For a GUV with a uniform membrane, the shape that minimizes the bending energy (Eq. 3.11) is found to be a sphere. If the membrane contains domains with different bending moduli κ , the sphere is no longer the optimal solution.

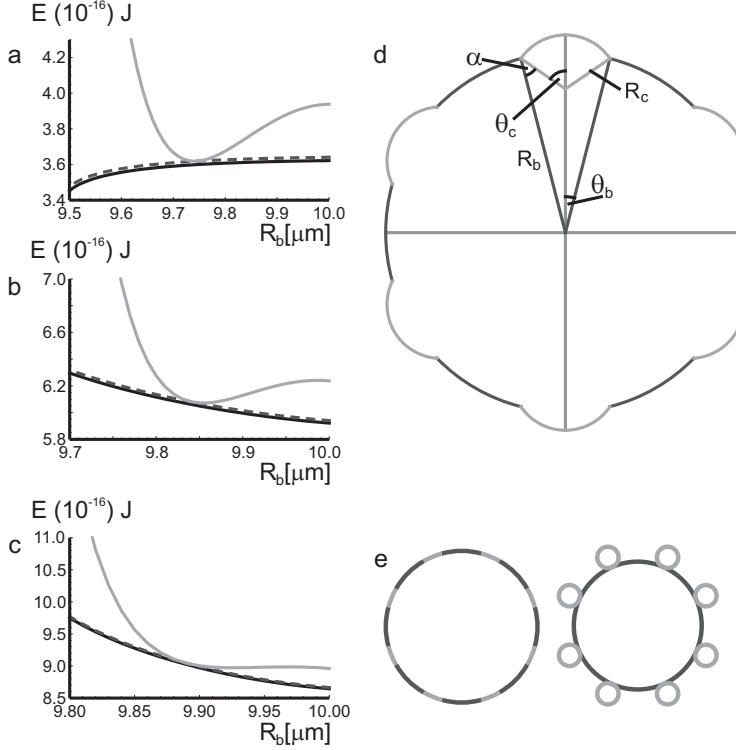


Figure 3.6

Energies of the sphere-with-domains system for 10 (a), 25 (b) and 50 (c) domains as a function of the radius R_b of the ‘background sphere’ in micrometers. In each case the geometrical (Eq. 3.15) and volume constraint (Eq. 3.16) are met and the total area of the domains is fixed. The vesicle has a surface to volume ratio that is slightly larger than that of a sphere, to create area for the domains to bud out. For the material parameters we use the values we obtained in an earlier study on phase-separated membrane vesicles (20) (Chap. 2 of this thesis). The black solid line shows just the contributions of curvature and line tension; the dashed line those plus a surface tension term, and the gray solid line all contributions including a surface elasticity term (Eq. 3.21). Without the surface elasticity term, the minimum of the energy is located at the maximum vesicle radius (figs. b and c), implying flat domains (fig. e left), or the minimum vesicle radius (fig. a), implying full budding (fig. e right). In the case of 50 domains the line tension is not strong enough yet to create buds, but when there are only 25 it forces the domains to bud out and deform the membrane around, halting or at least slowing down further fusion of domains. The energy without the surface elasticity term predicts that the buds form complete spheres, whereas the one with the surface elasticity predicts spherical caps, as observed. In these plots the excess area fraction $\frac{R_A - R_V}{R_V} = \left(\frac{A}{4\pi}\right)^{\frac{1}{2}} \left(\frac{3V}{4\pi}\right)^{-\frac{1}{3}} - 1$ is equal to 0.012. Figure d shows the coordinate system for the spherical caps model and figure e the two extremal situations - complete budding (right) and no budding at all (left).

However, within the ‘bulk’ of each domain, far away from any domain boundary, the sphere is still a good approximation of the actual membrane shape. For the case at hand, where we have many small and relatively stiff domains in a more flexible ‘background’, we follow Lipowsky (90) and model the small domains as spherical caps on a vesicle which also has spherical shape itself (see Fig. 3.6d). Although this model has the serious shortcoming that it suggests infinite curvature at the domain edge, it remains a good approximation for the overall vesicle shape, because it corresponds to the minimal-curvature solution of the shape equation on the entire vesicle except a few special points. For the special case that all domains are equal in size, we can describe them with a curvature radius R_c and opening angle θ_c , and the background sphere with its radius R_b and opening angle θ_b (see Fig. 3.6d). For the mean curvature energy of a system with N domains we then have

$$\mathcal{E}_{\text{curv}} = 4\pi\kappa_o N(1 - \cos \theta_c) + 4\pi\kappa_d(2 - N(1 - \cos \theta_b)), \quad (3.12)$$

where κ_o and κ_d are the bending moduli of the L_o and L_d phases respectively. The Gaussian curvature contribution is given by the boundary term

$$\mathcal{E}_{\text{Gauss}} = 2\pi N \Delta\kappa_G \cos \theta_c, \quad (3.13)$$

with $\Delta\kappa_G$ the difference in Gaussian curvature modulus between the L_o and L_d domains. As mentioned above, we model the fact that the lipids separate into two phases by assigning a line tension to the phase boundary. The energy associated with that line tension τ in the spherical cap model is given by

$$\mathcal{E}_{\text{tens}} = 2\pi\tau N R_b \sin \theta_b. \quad (3.14)$$

If the total number N of domains is fixed, the energy given by the sum of Eqs. 3.12, 3.13 and 3.14 is a function of four variables: R_b , R_c , θ_b , and θ_c . These variables are not independent, since they are subject to constraints. The first is that the membrane must be continuous at the domain boundary, which gives the geometric constraint

$$R_c \sin \theta_c = R_b \sin \theta_b. \quad (3.15)$$

Since the volume of the vesicle will change only over long timescales (hours) (176), we assume it is constant in our experiment (minutes), leading to a volume constraint on our system

$$\frac{4\pi}{3} [R_b^3 + N R_c^3 (1 - \cos \theta_c)^2 (2 + \cos \theta_c) - N R_b^3 (1 - \cos \theta_b)^2 (2 + \cos \theta_b)] = V_0, \quad (3.16)$$

where V_0 is the volume of the vesicle. Finally we consider the area of the vesicle. We have to treat the (total) area of the domains and that of the bulk phase separately. If we fix both of them, we obtain two additional constraints:

$$2\pi NR_c^2(1 - \cos \theta_c) = A_{c,0}, \quad (3.17)$$

and

$$2\pi R_b^2(2 - N(1 - \cos \theta_b)) = A_{b,0}. \quad (3.18)$$

If all four constraints given by Eqs. 3.15-3.18 are imposed rigorously, the shape of the vesicle is fixed, because there were only four unknowns in the system. For an experimental system at temperature $T > 0$ however, the total area is not conserved. Thermal fluctuations cause undulations in the membrane, resulting in a larger area than the projected area given by $A_{c,0}$ and $A_{b,0}$ (79). For $T > 0$ we should therefore not work in a fixed-area ensemble, but rather in a fixed surface-tension ensemble. We drop the constraints given by Eqs. 3.17 and 3.18 and instead add an ‘area energy’ term to the total energy

$$\mathcal{E}_{\text{area}} = 2\pi\sigma_o NR_c^2(1 - \cos \theta_c) + 2\pi\sigma_d R_b^2(2 - N(1 - \cos \theta_b)), \quad (3.19)$$

with σ_o and σ_d the surface tensions of the L_o and L_d phases respectively. Note that Eq. 3.19 can be interpreted in two ways: in the fixed area ensemble, it contains two freely adjustable Lagrange-multipliers (σ_o and σ_d) which enforce the conditions given by Eqs. 3.17 and 3.18. In the fixed surface tension ensemble, σ_o and σ_d are set and the shape is found by minimizing the total energy with respect to the free parameters, considering the remaining geometrical and volume constraints given by Eqs. 3.15 and 3.16. These constraints can of course be included in the total energy using Lagrange multipliers as well. This is often done for the volume constraint, and the associated Lagrange multiplier is usually identified as the pressure difference across the membrane. We stress that since we fix the total volume (i.e., work in a fixed volume ensemble), this pressure is selected by the system and is not an input parameter. The Lagrange-multiplier approach is mathematically equivalent to imposing an external volume constraint as we do here for practical purposes.

Eq. 3.19 correctly gives the free energy contribution of the ‘area energy’ in what is called the entropic regime, where the dominant contribution to the area term is due to the thermal fluctuations of the membrane (79). To account for the fact that the membrane itself can be stretched or

compressed away from its ‘natural area’ A_0 , we include a quadratic term in the area of the membrane (177)

$$\mathcal{E}_{\text{el}} = \gamma \left(\frac{A - A_0}{A_0} \right)^2. \quad (3.20)$$

The elastic modulus γ is approximately 10^{-14} J in the tricomponent system considered here (176). One way to understand Eq. 3.20 is that in the high-tension or elastic regime, the surface tension is no longer a fixed number, but itself depends linearly on the area (79). The total shape energy is given by the sum of the five contributions (Eqs. 3.12, 3.13, 3.14, 3.19, 3.20)

$$\mathcal{E} = \mathcal{E}_{\text{curv}} + \mathcal{E}_{\text{Gauss}} + \mathcal{E}_{\text{tens}} + \mathcal{E}_{\text{area}} + \mathcal{E}_{\text{el}}. \quad (3.21)$$

With the constraints (Eq. 3.15) and (Eq. 3.16), we are left with two independent variables for the minimization of the total energy. Since the surface tension and elastic modulus of the L_o phase are much larger than that of the L_d phase (20, 176) (Chap. 2 of this thesis), we further assume that the area of the L_o domains is fixed. This leaves us with a single variable minimization problem, which we solve numerically. For the material parameters we use the values we obtained in an earlier study on phase-separated membrane vesicles (20) (Chap. 2 of this thesis). The results are shown in Fig. 3.6. In the same figure we plot the energy without the membrane stretching term (Eq. 3.20). In this case we find no partial budding, showing that the area elasticity term is required to reproduce the experimental results, and that our experimental vesicles are well within the elastic regime. Plotting the minima of the energy as a function of the number of budded domains on the vesicle, we find that it decreases with the number of domains (Fig. 3.7). Therefore the fully phase-separated vesicle is the ground state, as we expected from the fact that the line tension is strong enough to dominate the shape.

3.5 Measuring the interactions

3.5.1 Domain position tracking

In order to determine quantitatively the interaction strength between the domains, we tracked their positions over time. In particular, we regarded situations like the one shown in Fig. 3.8, in which a single domain is surrounded and held in place by a shell of 4 to 6 neighbor domains. We

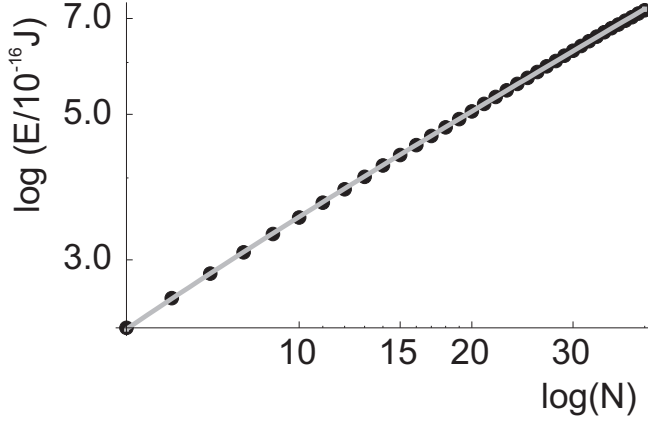


Figure 3.7

Minimum of the energy (Eq. 3.21 in the main text) for a given number of domains N . Since the line tension contribution is the largest, decreasing the number of domains (and hence shortening the total domain boundary) lowers the energy, in correspondence with the result that the fully phase-separated vesicle is the ground state. From the logarithmic plot shown here we find that the total energy as a function of the number of domains behaves as a power law with exponent 0.53 (solid gray line).

recorded the distance between the central domain and the center of mass of the shell domains (projected on the vesicle surface) over time and calculated the mean squared displacement (msd), see Fig. 3.8 for a typical example. Using only relative distances eliminates any influence of putative flow or overall movement of domains.

Although the precise form of the potential that confines the central domain is not known, we can approximate it around the local minimum by a harmonic potential $U(x) = \frac{1}{2}kx^2$ with spring constant k . If we treat the domain as a random walker with diffusion constant D , our model is formally equivalent to an Ornstein-Uhlenbeck process (178). Alternatively, one can imagine all domains connected by harmonic springs. This approach also leads to an isotropic harmonic confining potential for the central domain. The msd of the domain is given by:

$$\langle \Delta x^2(\Delta t) \rangle = \frac{4k_B T}{k} \left[1 - \exp\left(-\frac{kD}{k_B T} \Delta t\right) \right] \approx 4D\Delta t \text{ for small } \Delta t \quad (3.22)$$

In practice we determined the diffusion coefficient D (and a small offset due to the finite positional accuracy) from a linear fit to the first 3 time

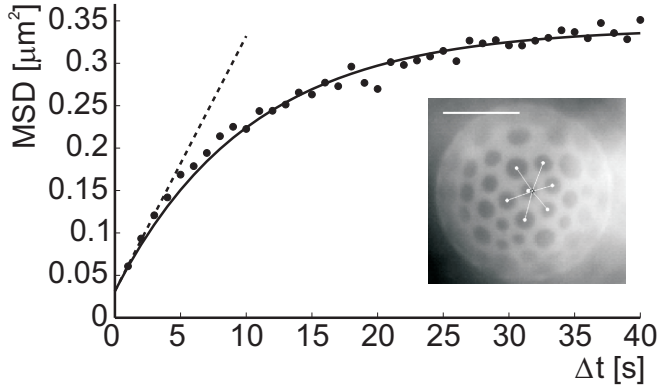


Figure 3.8

Typical example of the mean square displacement (*msd*) of the distance between central domain and center of mass of the surrounding domains (dots); solid line: fit to the Ornstein-Uhlenbeck model (Eq. 3.22); dashed line: linear fit to the first three data points (dashed line). Inset: Example for tracking configuration. White dots: centroids of domains; black dot: center of mass of domains constituting the shell; gray line: vector connecting the centroid of the central domain and the center of mass of the shell domains. The mean square displacement of this distance is used to determine the diffusional behavior of the central domain. Scalebar 20 μm .

lags (see Fig. 3.8), since the reliability of the data points is highest in that region. Fig. 3.9 shows the diffusion coefficient as a function of the size of the central domain.

The diffusion coefficient depends only very weakly on the domain size r . With micrometer sized, liquid ordered domains in a liquid disordered background (with a sufficiently small membrane viscosity) one would expect the diffusion coefficient to follow (179):

$$D(r) = \frac{k_B T}{16\eta} \frac{1}{r} \quad (3.23)$$

where $\eta \approx 10^{-3} \text{ Ns/m}^2$ is the bulk viscosity of water (see dashed line in Fig. 3.9). This relation holds if the friction between the domain and the surrounding water is big compared to the friction between domain and surrounding membrane. Therefore the 2D membrane viscosity η' does not play a role and does not appear in Eq. 3.23. The fact that the measured diffusion coefficients are significantly smaller than predicted by Eq. 3.23 shows that either the membrane friction is much higher than expected or

the interaction between the domains has some influence on the diffusion coefficient.

In order to determine the 2D membrane viscosity η' we employ the Hughes-Pailthorpe-White (180) model for the radius-dependent diffusion constant $D(r)$ which is valid for arbitrary domain size r , water viscosity η and membrane viscosity η' . Instead of the exact form of this model we use an approximation due to Petrov and Schwille (181). By fitting this approximation to the data (thick solid line in Fig. 3.9) we find the 2D membrane viscosity to be $\eta' = 4.8 \times 10^{-8}$ Ns/m. This value is comparable to the values found in (179) where the diffusion of unbudded, liquid disordered domains in a liquid ordered background is measured.

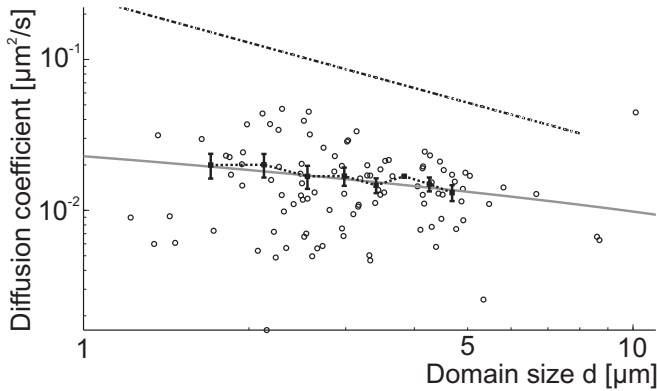


Figure 3.9

Diffusion coefficient versus domain radius (circles) for 103 trajectories. The squares represent binned data. For comparison, the dashed-dotted line gives the behavior of $D(r)$ according to Eq. 3.23 if the viscosity of water is dominant. The gray solid line shows a fit to the model described in (181) which gives $\eta' = 4.8 \times 10^{-8}$ Ns/m for the 2D membrane viscosity. Reported error bars are standard errors of the mean.

The other parameter of the Ornstein-Uhlenbeck model for the msd of a domain (Eq. (3.22)) is the spring constant k . We determined its value from a fit of Eq. (3.22) to the full experimental data set, where D was fixed to the value determined before. Fig. 3.10 shows k normalized by the number of nearest neighbors as a function of the size of the central domain. On average $k = 1.4 \pm 0.5 k_B T / \mu\text{m}^2$. This value supports the observation that domains are stable over extended periods of time: since the distance between domains is typically several μm the energy barrier that the domains have to overcome in order to fuse is well above $k_B T$. Due to the

limited amount of available trajectories, the error in the determination of k is fairly large. Hence it is not possible to deduce the quantitative dependence of k on the domain size. Therefore we determined k more precisely in a separate, independent way, based on domain distance statistics.

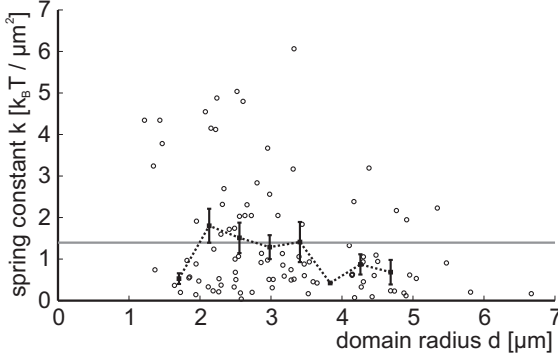


Figure 3.10

Spring constant k corrected for the number of nearest neighbors versus domain radius (circles), the squares correspond to binned data. The gray solid line marks the average $k = 1.4 \pm 0.5 k_B T / \mu\text{m}^2$. Reported error bars are standard errors of the mean.

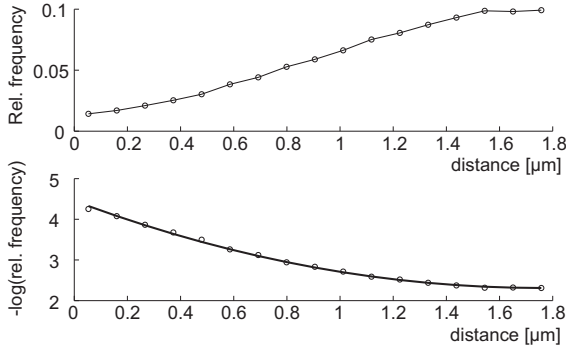
3.5.2 Domain distance statistics

The interaction potential between two domains can be directly inferred from the distribution of domain distances, as already demonstrated by Rozovsky et al. (87). We consider a central domain surrounded by N nearest neighbors, whose combined imposed potential is given by U . Then the probability $p(x)$ to find the central domain a distance x from the center of mass of the neighbors is proportional to the Boltzmann factor $p(x) \propto \exp\left(-\frac{U(x)}{k_B T}\right)$. As before we assume the imposed potential, at least locally, to be harmonic, $U(x) = \frac{1}{2}kx^2$, which gives for $p(x)$:

$$-\ln(p(x)) = \text{const.} + \frac{1}{2}kx^2. \quad (3.24)$$

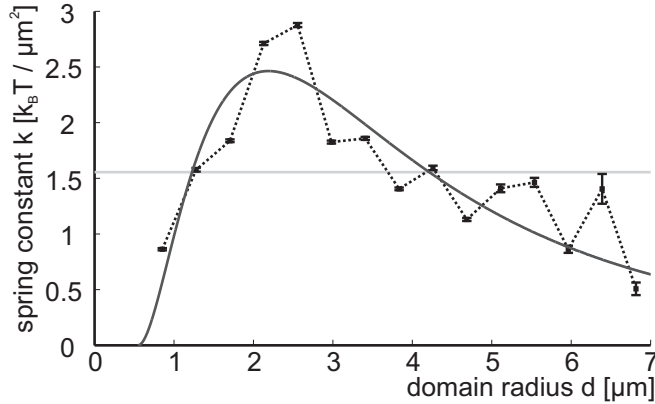
In order to determine k , we used Eq. 3.24 to fit $-\ln(p(x))$, where $p(x)$ was determined from the distances of the 4 nearest neighbors of each domain. When determining $p(x)$, the data was binned according to the size of the central domain. Fig. 3.11 shows an example of the distance distribution and a fit of the potential to $-\ln(p(x))$.

The available data set for domain distances is much larger than the one we obtained from domain tracking. Consequently, the spring constant k can be determined with a smaller error, see Fig. 3.12. The average $k =$

**Figure 3.11**

Spring constant determined by domain distance statistics. Upper plot: relative frequency of edge-edge distances; lower plot: $-\log(\text{rel. frequency})$ with fit to harmonic potential (solid line).

$1.6 \pm 0.2 k_B T / \mu\text{m}^2$ coincides with the result found from domain tracking $k = 1.4 \pm 0.5 k_B T / \mu\text{m}^2$. Interestingly, k shows a nonlinear behavior with a clear maximum for domains of an intermediate size which roughly coincides with the size of the most abundant domains, see Fig. 3.3.

**Figure 3.12**

Effective spring constant k versus domain radius (circles), the squares correspond to binned data. The light gray solid line marks the average $k = 1.6 \pm 0.2 k_B T / \mu\text{m}^2$ and the dark gray solid line the theoretical fit (determined using Eq. 3.29).

Due to the fact that the membrane of a GUV is both curved and finite in size, the calculation of the interaction potential between two distortions on such a membrane is a very difficult task. However, in the case where we are dealing with a large number of small domains on a big vesicle the situation approaches that of domains on an infinite and asymptotically

flat membrane. For two such domains with the shape of spherical caps, the interaction potential was first calculated by Goulian et al. (54) and reads

$$V = 4\pi\kappa(\alpha_1^2 + \alpha_2^2) \left(\frac{a}{r}\right)^4 \quad (3.25)$$

where r is the center-to-center distance between the two domains, a is a cutoff lengthscale taken to be the membrane thickness (a few nanometers), α_1 and α_2 are the domain's contact angles with the surrounding membrane (see Fig. 3.6d) and κ is the bending modulus of the 'background' membrane. The domains themselves are again assumed to be nondeformable spherical caps, which is a good approximation given that the ratio of their bending modulus with that of the surrounding membrane is significantly larger than 1 ($\frac{\kappa_o}{\kappa_d} \approx 4$) (20) (Chap. 2 of this thesis).

As Dommersnes and Fournier showed (172), the interaction between multiple inclusions is not equal to the sum of their pairwise interactions. However, the scaling of the interaction with the distance between the domains r and the contact angles α_i does not change, only the prefactor does. For any budded domain surrounded by several other budded domains, we can therefore assume a potential of the form

$$V = \bar{C}\kappa a^4 \sum_{i=1}^N \frac{\alpha_0^2 + \alpha_i^2}{r_{0i}^4}, \quad (3.26)$$

where $\bar{C}$ is an (unknown) numerical constant, α_0 the contact angle of the domain we are interested in, α_i that of the i th neighbor and r_{0i} the distance between the 'central' domain and its i th neighbor. The number of neighbors is N , which in experimental vesicles is typically 5 or 6, corresponding to a relatively dense packing of domains. Let us assume for simplicity that the equilibrium of the potential (Eq. 3.26) is such that the nearest neighbors form a circle of radius r_0 around it, on which they are on average equally distributed. This mean field assumption means that the central domain sees its environment as isotropic (it is not pushed in any particular direction) and its potential has a unique global minimum at the center of the circle. The energy of any displacement Δr of the central domain away from its energy minimum can then be calculated by an expansion in Δr of Eq. 3.26. The linear term in that expansion vanishes because of the isotropic distribution of the neighbors, in agreement with the assumption of the existence of a global potential minimum at $\Delta r = 0$. The first term of interest is therefore the quadratic term, which is given

by

$$V_{\text{quadratic}} = \frac{C\kappa a^4}{2} \frac{\alpha_0^2 + \beta^2}{r_0^6} (\Delta r)^2 \quad (3.27)$$

where C is another constant and β the contact angle of a neighboring domain that would correspond to the time-average isotropic potential assumed above. Eq. 3.27 allows us to experimentally determine the strength of the interactions between budded domains, since it yields an effective spring constant which can be measured

$$k = C\kappa a^4 \frac{\alpha_0^2 + \beta^2}{r_0^6}. \quad (3.28)$$

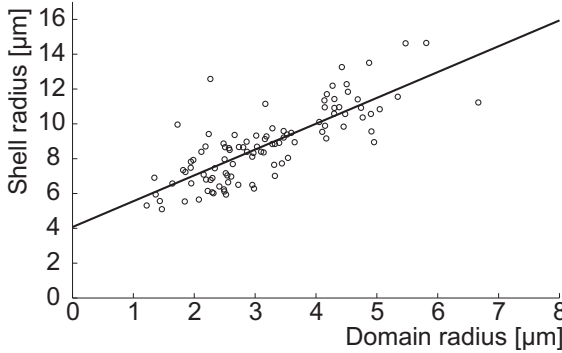
In order to be able to predict the behavior of the spring constant k as a function of the domain size d (the length of its projected radius), we need to establish how α and r_0 vary with d . At present we have no way of determining $\alpha(d)$ from first principles, since that would require having a full description of the complete vesicle membrane. We can argue though that at least it should be an increasing function of d for small domains. When a domain has just grown large enough to ‘bud out’, its circumference will still be small, and the amount of membrane bending and stretching it can induce to reduce the line tension term will also be small. As the domain grows in size, this balance shifts and by budding out further it makes its presence felt more strongly in the surrounding membrane. Because in our experimental system we always consider vesicles with many small domains, we assume $\alpha(d)$ to be in the linear regime. We therefore phenomenologically write: $\alpha \propto (d - d_0)$, where d_0 is the domain size at which budding first occurs, which should be of the order of the invagination length (0.5-1.0 μm , see Sec. 3.4).

For $r_0(d)$ we do not need to make a guess, but can simply rely on experimental results, which show that r_0 depends linearly on d (Fig. 3.13).

Finally we will assume that $\alpha_0 \sim \beta$, since in experiments we always find that domains are typically surrounded by domains of approximately equal size (56). Using the linear dependencies of α_0 and r_0 on d in the expression for the spring constant (Eq. 3.28), we find

$$k = A \frac{(d - d_0)^2}{(\bar{r}_0 + cd)^6}. \quad (3.29)$$

Eq. 3.29 has two fitting parameters (A and d_0). The best fit of the experimental data is given by the dark gray solid line in Fig. 3.12. We find

**Figure 3.13**

Shell radius versus central domain radius, the solid line corresponds to a linear fit with slope 1.5 and offset 4.1 μm.

$A = 1.5 \times 10^5 k_B T \mu\text{m}^2$ and $d_0 = 0.55 \mu\text{m}$, which indeed is approximately the size of the invagination length ($0.7 \mu\text{m}$). Qualitatively we find that due to the increase in repulsion strength with growing domain size the spring constant increases with domain size for small domains. For very large domains on the other hand the interdomain distance also grows, and because the interactions fall off very steeply with distance, the spring constant decreases. In between we find a maximum that corresponds to the most abundantly present domain size in the experimental vesicles.

3.6 Conclusion

We have experimentally demonstrated the presence of membrane mediated interactions in lipid bilayer membranes and quantified their strength. We have shown that these interactions originate in locally imposed curvature from the domains on their immediate environment. We have also shown that the phenomenon of ‘partial domain budding’ can be explained as a competition between curvature and elastic forces on the one hand and tensile forces on the other hand. Furthermore we found that the membrane mediated interaction influences the fusion behavior of domains, resulting in a preferred domain size. Using a simple Monte Carlo simulation we were able to reproduce the experimental domain size distribution. Finally we found that the dependence of the interaction strength on distance is consistent with existing theory, which gives a $1/r^4$ dependence.

Proteins in the membranes of living cells distort their surrounding membrane in the same fashion as lipid domains do. We therefore predict that similar membrane mediated interaction forces play a significant role

in membrane structuring. Coarse grained simulations show that membrane mediated interactions can lead to the aggregation of membrane inclusions (53). In our experiments we do not observe such attractive behavior, which suggests that our model system is more comparable to larger structures, like protein aggregates. We expect that such aggregates experience repulsive interaction if they impose a curvature on the membrane. If this curvature exceeds a certain critical size the aggregates will not be able to grow further, just like the domains stop growing after reaching a certain size. Moreover, the membrane mediated interaction reported here has a longer range ($1/r^4$) than van der Waals interactions ($1/r^6$) and should therefore be the dominant interaction effect in the absence of electrical charges. We therefore expect this interaction to play an important role in many biological processes.

CHAPTER 4

MEMBRANE MEDIATED SORTING

Inclusions in biological membranes may communicate via deformations they induce on the shape of that very membrane, a purely physical effect which is not dependent on any specific interactions. In this paper we show that this type of interactions can organize membrane domains and proteins and hence may play an important biological role. Using a simple analytical model we predict that membrane inclusions sort according to the curvature they impose. We verify this prediction by both numerical simulations and experimental observations of membrane domains in phase separated vesicles.

4.1 Introduction

On the mesoscopic scale, cellular organization is governed by some well known forces: hydrophobic, electrostatic and van der Waals interactions (170). These forces are responsible for the structure of the lipid bilayer and highly specific protein-protein interactions. However, due to their short range, they do not provide a mechanism for some important biological functions like the recruitment of proteins to certain regions in the plasma membrane. Recently attention was drawn to another type of interaction: membrane curvature mediated interactions (42, 43, 53–55, 172, 182, 183). These interactions are known to be long ranged (54) and non-pairwise additive (172). In this paper we demonstrate how membrane mediated interactions give rise to long range order in a biomimetic system. In the membranes of living cells the breaking of the homogeneity by the formation of patterns and long-range order carries significant biological implications for processes like signaling, chemotaxis, exocytosis and cell division.

A well suited system to study membrane mediated interactions is a giant unilamellar vesicle (GUV) composed of two types of lipids and cholesterol. For many different compositions such GUVs phase separate into liquid ordered (L_o) and liquid disordered (L_d) domains (3, 60, 70). Phase separation is driven by a line tension on the boundary between the two phases (20, 22, 71) (Chap. 2 of this thesis). Typically one finds many domains of one phase on a ‘background’ vesicle of the other phase. The line tension causes the domains to be circular in shape. Moreover, domains sometimes partially ‘bud out’ from the spherical vesicle to reduce the boundary length even further (70).

Recent experiments have shown that partially budded membrane domains repel due to membrane mediated interactions (70, 88, 89) (Chap. 3 of this thesis). From measurements of domain fusion dynamics (88) and the distribution of domain sizes (89) (Chap. 3 of this thesis) it became evident that membrane mediated interactions require a minimum domain size. If domains are too small their curvature equals that of the surrounding membrane. In that case the line tension between the L_o and L_d phase (20) (Chap. 2 of this thesis) cannot push the domains out of the background membrane. Consequently, domains do not experience any curvature related interaction (89) (Chap. 3 of this thesis). As the domain circumference grows due to repeated fusion events the influence of the line tension eventually becomes bigger than that of the bending rigidity and

the domain partially buds out. This leads to a repulsive interaction that increases with domain size. Consequently, domain coalescence slows down significantly after reaching a certain preferred size (88, 89, 184) (Chap. 3 of this thesis). This preferred size can be found as a maximum in the domain size distribution. Although the domains no longer coalesce, they are by no means static, but rather mobile and reorganize continuously. The interaction strength decays with interdomain distance as $1/r^4$ (54). Because larger domains exert a greater force on their neighbors, the domains will collectively try to find a configuration in which larger domains have a larger effective area around them. We expect that, due to this size-dependent interaction, the domains demix by size to achieve an optimal configuration.

We note that this effect is different from depletion interaction in the sense that the distribution of domain sizes in our system is narrow. Moreover, the interaction we consider here is both long ranged and soft, whereas depletion is an effect seen in systems with hard-core repulsions. Depletion may of course still play a small role, but can be ignored in comparison to the membrane mediated interactions discussed here.

In this paper we present an analytical model in which we analyze the possible distributions of domains on phase separated vesicles, and find that they exhibit a striking tendency to sort. We complement this model by performing both Monte Carlo and molecular dynamics simulations using realistic membrane parameters that were experimentally obtained in previous work. The simulations give the optimal domain distribution and show the sorting effect. We find that sorting is an unavoidable consequence of the size-dependent nature of the interactions and the finite area available on a vesicle. In addition, we present experimental results on lipid vesicles composed of two types of lipids and cholesterol, which do indeed show the sorting effect. In particular, we find a correlation between the size of a domain and the size of its neighbors, which is reproduced by our simulations.

4.2 Materials and Methods

The experimental procedures were described in detail in (89) (Chap. 3 of this thesis). Briefly, giant unilamellar vesicles (GUVs) were produced by electroformation in a flow chamber (62, 64) from a mixture of 30 % DOPC, 50 % brain sphingomyelin, and 20 % cholesterol at 55 °C. The liquid dis-

ordered L_d phase was stained by a small amount of Rhodamine-DOPE (0.2 % - 0.4 %), the liquid ordered L_o with a small amount (0.2 % - 0.4 %) of perylene. The DOPC (1,2-di-oleoyl-sn-glycero-3-phosphocholine), sphingomyelin, cholesterol and Rhodamine-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl)) were obtained from Avanti Polar Lipids; the perylene from Sigma-Aldrich. To stimulate the partial budding of domains, the osmolarity on the outside of the vesicles was increased by 40-50 mM. Lowering the temperature to 20 °C resulted in the spontaneous nucleation of partially budded liquid ordered L_o domains in a liquid disordered L_d matrix. Those domains possess long term stability (time scale of hours). The movement of domains was monitored in time with an inverted wide-field fluorescence microscope (Axiovert 40 CFL, Zeiss). Proper filtering and analysis of the raw images yielded the sizes of all domains and their movement in time.

4.3 Analytical model

A somewhat oversimplified analysis of the total energy of a fully mixed and a fully demixed system gives us a direct clue as to whether the domains segregate into regions of identical-sized ones or not. Because the bending rigidity of the L_o domains is much higher than that of the L_d background (20) (Chap. 2 of this thesis), we assume the domains to be rigid inclusions. As was first shown by Goulian *et al.* (54) there is a repulsive potential between two inclusions in an infinite membrane that drops off as $1/r^4$, with r the distance between the inclusions. Moreover, the interaction strength depends on the imposed contact angle at the edge of an inclusion, and for two inclusions with contact angles α and β we have

$$V \sim \frac{\alpha^2 + \beta^2}{r^4}. \quad (4.1)$$

Although the interactions are not pairwise additive, the qualitative dependence of V on the contact angles and inclusion distance does not change if more inclusions are added to the system (172). It is therefore possible to use a mean-field description for a finite, closed system with many inclusions, from which the prefactor in equation (4.1) can be determined experimentally (89) (Chap. 3 of this thesis). Moreover, we can write effective pairwise interactions for nearest-neighbor domains based on their size, as a function of their distance.

For simplicity we look at a system with only two sizes of domains, which we will call ‘big’ and ‘small’. This choice is motivated by earlier experimental results that show a narrow distribution of domain sizes (89) (Chap. 3 of this thesis). In our model the most abundant experimental domain size (with a typical radius of $3.0 \mu\text{m}$) corresponds to the small domains. For the big domains we take a radius of $(3.0 \mu\text{m}) \cdot \sqrt{2} = 4.3 \mu\text{m}$, which means that their area is twice that of the small domains.

Let us denote the number of domains by N , the number of big domains by $N_b = \gamma N$ and that of small domains by $N_s = N - N_b = (1 - \gamma)N$. Likewise we denote the contact angle of a big domain by α_b , that of a small domain by α_s , and the average contact angle of a domain’s nearest neighbors (in the mean-field approach) by β . If we neglect the small curvature of the background sphere, which has surface area A , we can associate an ‘effective radius’ to each domain corresponding to the patch of area which it dominates (i.e., in which it is the closest domain). In a completely mixed system the effective radius of all domains is equal and given by $R_{\text{eff}} = \sqrt{A/(\pi N)}$. In a fully mixed system each of the domains has $6 \cdot \gamma$ big and $6 \cdot (1 - \gamma)$ small neighbors, which allows us to calculate the potential of that configuration in the mean field approach:

$$V_{\text{mixed}} = \frac{6}{16} N_b \frac{\alpha_b^2 + \beta^2}{A^2/(\pi^2 N^2)} + \frac{6}{16} N_s \frac{\alpha_s^2 + \beta^2}{A^2/(\pi^2 N^2)} \quad (4.2)$$

where $\beta = \gamma \alpha_b + (1 - \gamma) \alpha_s$. In the fully demixed system, the big domains can take up a larger fraction ϕ of the vesicle surface than they occupy in the fully mixed system. By doing so they can increase the distance between them, reducing the interaction energy. The penalty for this reduction is a denser packing of the small domains, but since their repulsive forces are smaller, the total configuration energy can be smaller than in the mixed system. We consider the regions in which we have big and small domains separately and get two effective radii: $R_{\text{eff}}^b = \sqrt{(\phi A)/(\pi N_b)}$ and $R_{\text{eff}}^s = \sqrt{((1 - \phi) A)/(\pi N_s)}$. For the potential energy we obtain:

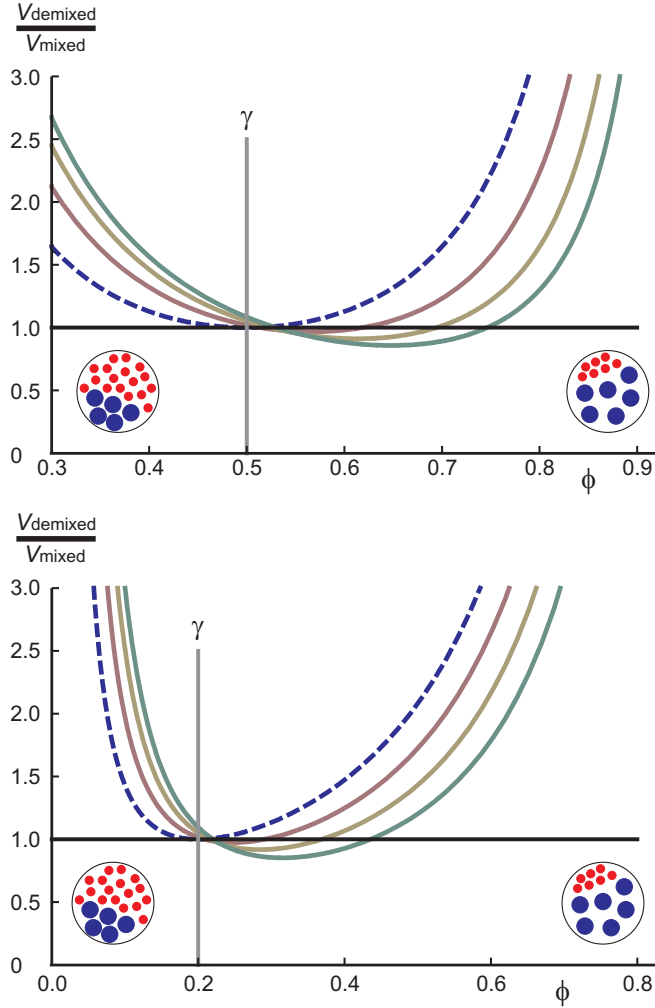
$$V_{\text{demixed}} = \frac{6}{16} N_b \frac{2\alpha_b^2}{(\phi A/(\pi N_b))^2} + \frac{6}{16} N_s \frac{2\alpha_s^2}{((1 - \phi) A/(\pi N_s))^2} \quad (4.3)$$

where we have assumed the number of domains is large enough that ignoring the boundary between the two regions is justified. For a fully mixed

system we would have $\phi = \gamma$, or the area fraction assigned to the big domains is equal to their number fraction. In the demixed system the parameter ϕ becomes freely adjustable and can be tuned to minimize the interaction energy. Comparing the demixed potential Eq. 4.3 to the mixed potential Eq. 4.2, we find

$$\begin{aligned} \frac{V_{\text{demixed}}}{V_{\text{mixed}}} = & 2 \left[\frac{\gamma^3}{\phi^2} \left(\frac{\alpha_b}{\alpha_s} \right)^2 + \frac{(1-\gamma)^3}{(1-\phi)^2} \right] \\ & \cdot \left[\gamma(1+\gamma) \left(\frac{\alpha_b}{\alpha_s} \right)^2 + 2\gamma(1-\gamma) \left(\frac{\alpha_b}{\alpha_s} \right) \right. \\ & \left. + (1-\gamma)(2-\gamma) \right]^{-1}. \end{aligned} \quad (4.4)$$

Plots for several values of the parameters are given in Fig. 4.1. For a range of values of the adjustable parameter ϕ the energy of the demixed state is smaller than that of the mixed state; this effect becomes more pronounced as the difference in contact angle (and therefore repulsive force) increases. In the configuration which has the lowest total energy the area fraction ϕ claimed by the big domains is indeed larger than their number fraction γ .

**Figure 4.1**

Comparison of the potential energies of the completely mixed and completely demixed state of a vesicle with domains of two different sizes. The freely adjustable parameter ϕ denotes the fraction of the vesicle's surface area claimed by the big domains. The top figure has $\gamma = \frac{1}{2}$ (equal numbers of big and small domains), and the bottom figure has $\gamma = \frac{1}{5}$ (one fifth of the domains is big). The dashed blue line indicates the case in which the 'big' and 'small' domains are equal in size (and hence have equal contact angles). The solid red, yellow and green lines indicate contact angle ratios α_b/α_s of 1.5, 2.0 and 2.5 respectively. Domain demixing occurs for any value of ϕ for which the potential ratio is less than 1 (black horizontal line). For comparison the number fraction γ of the big domains is indicated by the gray vertical line. Insets: typical distributions of domains for small (left) and big (right) values of ϕ . For small ϕ , the big domains are packed closely together and the small domains claim the largest area fraction, for large ϕ the situation is reversed.

4.4 Simulations

In the analytical model we only considered the two extreme configurations of a completely mixed and a completely demixed system. In order to be able to study also intermediate states of the system we performed Monte Carlo simulations in which we included all nearest-neighbor interactions. In these simulations we again studied a binary system consisting of small and big domains, where the surface area of the big domains is twice that of the small ones. Starting from a random configuration of big and small L_o domains on a L_d sphere, we used Monte Carlo steps to find the energy minimum, and consistently found demixing. A typical example of a relaxation process and a configuration after 50,000 timesteps are shown in Fig. 4.2.

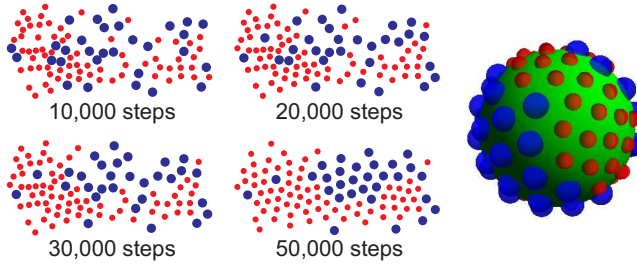


Figure 4.2

Monte Carlo relaxation of a random configuration of 70 small (red) and 30 big (blue) domains on a spherical vesicle. Left: a folded-open view of the entire vesicle, with the azimuthal angle along the horizontal direction and the polar angle along the vertical direction. The configuration is shown after 10,000 (top left), 20,000 (top right), 30,000 (bottom left) and 50,000 (bottom right) timesteps. Here $V_{s-b} = 3.3V_{s-s}$, $V_{b-b} = 4.5V_{s-s}$ and $k_B T = 0.25V_{s-s}$. Right: the configuration on a sphere after 50,000 timesteps.

Complementing the Monte Carlo simulations, we also performed molecular dynamics simulations. In these simulations, we calculate in each timestep the force on each domain due to its nearest neighbors and displace it accordingly. Moreover, we add thermal fluctuations by displacing each domain a distance x over an angle θ in each timestep. The angles are sampled from a uniform distribution and the distances are sampled from the distribution $P(x) \sim \exp\left(-\frac{kx^2}{2k_B T}\right)$, where k is the effective spring constant due to the potential created by a domain's nearest neighbors. A typical value for this spring constant is $1.5 k_B T / \mu\text{m}^2$ (89) (Chap. 3 of

this thesis). In these simulations, we do not just study a binary system but also a system with an exponential distribution of domain sizes (89) (Chap. 3 of this thesis). Including multiple domain sizes allows for better comparison with experiment; in particular we can look for correlations between the size of a domain and its nearest neighbors. The molecular dynamics simulations showed demixing like the Monte Carlo simulations did. An example of an obtained correlation plot is shown in Fig. 4.3a.

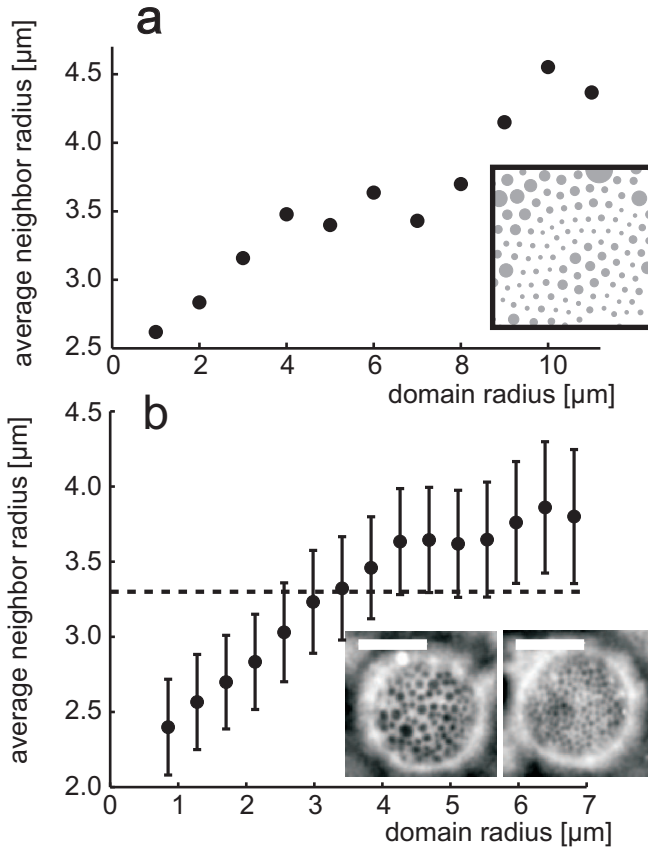


Figure 4.3

Correlations between the size of a domain and that of its nearest neighbors. a) Typical example of a correlation plot from a molecular dynamics simulation. Inset: Actual distribution of domains (gray) on the vesicle. b) Correlation plot averaged over 21 experimental vesicles; the dashed line corresponds to the average 3.3 μm. Inset: Two sides of the same vesicle showing very different domain sizes. Scalebar 20 μm.

4.5 Experimental verification

Our theoretical prediction that domains segregate into regions of equal-sized ones is confirmed by experimental observations. In experiments detailed in (89) (Chap. 3 of this thesis) we observed that big and small budded domains on the same vesicle tend to demix. Vesicles clearly have regions where some domain sizes are overrepresented. An example of such an experiment is given in the insets of Fig. 4.3b, where two sides of the same vesicle are shown. Quantitatively we found that there is a correlation between the size of a domain and the average size of its nearest neighbors (Fig. 4.3b). The domain sorting occurred consistently in all 21 vesicles with budded domains we studied.

4.6 Conclusion

As we have shown in this paper, membrane mediated interactions on closed vesicles lead to the sorting of domains by size. Our analysis shows that this is due to the fact that larger domains impose a larger curvature on their surrounding membrane. We expect the same sorting effect to occur for other curvature inducing membrane inclusions, in particular cone shaped transmembrane proteins. This spontaneous sorting mechanism could potentially be used to create polarized soft particles. Moreover, similar sorting effects may occur in the membranes of living systems without the need of a specific interaction or an actively driven process.

CHAPTER 5

PARTICLE IMAGE CORRELATION SPECTROSCOPY (PICS) RETRIEVING NANOMETER-SCALE CORRELATIONS FROM HIGH-DENSITY SINGLE-MOLECULE POSITION DATA¹

A new data analysis tool that resolves correlations on the nanometer length and millisecond time scale is derived. This tool, adapted from methods of spatiotemporal image correlation spectroscopy, exploits the high positional accuracy of single-particle tracking. While conventional tracking methods break down if multiple particle trajectories intersect, our method works in principle for arbitrarily large molecule densities and diffusion coefficients as long as individual molecules can be identified. We demonstrate the validity of the method by Monte Carlo simulations and by application to single-molecule tracking data of membrane-anchored proteins in live cells. The results faithfully reproduce those obtained by conventional tracking.

¹This chapter is based on: S. Semrau, T. Schmidt, Particle image correlation spectroscopy (PICS): Retrieving nanometer-scale correlations from high-density single-molecule position data, *Biophys. J.*, **92**, 613-621, (2007)

5.1 Introduction

Single-particle tracking (SPT) and image correlation microscopy (ICM) have been proven to be powerful tools for the investigation of local inhomogeneities in biological systems (4, 113, 185–188). Driven by recent discussions on the refinement of the classical fluid-mosaic model of the plasma membrane organization (1) both tools were applied to elucidate the contribution of lipid organization and protein interactions to the spatial organization of signaling molecules both *in vitro* and *in vivo*. Several structures have been suggested to influence the dynamics of membrane proteins; among these are clathrin coated pits, caveolae, lipid rafts and the cytoskeleton. Lipid rafts, especially, have been heavily discussed as possible organizational platforms for molecules involved in cell signaling (9). Their existence and the actual order of lipids in the plasma membrane is, however, still debated (18, 19, 157, 189). Recent studies have revealed that protein-protein interactions may play an important role in the spatial organization of signaling proteins (28, 190).

Single-particle tracking is ideally suited to study the dynamics of membrane molecules as this method is able to locate optical probes with a high positional accuracy down to a few *nm*. While gold nanoparticles and fluorescent quantum dots, being relatively large, allow for extremely long observation times (104, 105, 113, 185), labeling of proteins with fluorophores like e.g. eGFP or Cy5 is more suitable for biological applications. Those fluorophores, however, suffer from photobleaching. Therefore, tracking of individual molecules results in comparatively short trajectories (typically 10 steps) which makes the retrieval of individual trajectory dynamical information exceedingly difficult. However, given that the biological system is quite stable, the number of observations obtained under the same conditions can be large, to enable determination of dynamic properties of membranes in great detail (131).

For the implementation of SPT some *a priori* knowledge about the expected molecular behavior is needed since algorithms have to cope with the probabilistic nature of the tracking problem (113, 114). This is especially a drawback for data taken at higher concentrations, where molecular trajectories can accidentally be mixed. Image correlation microscopy (ICM) (187) and fluorescence correlation spectroscopy (113, 186) do not need any such prior information. However, both are regular imaging techniques limited in resolution by diffraction and thus a spatial resolution of 200 – 300 *nm*.

To overcome the drawbacks of both SPT and ICM we have developed a robust analysis method that combines both techniques. The method is self-contained on any ensemble of diffusion steps and therefore does not need individual traces to be assigned like in SPT. Consequently it can deal with arbitrarily high molecule densities and diffusion constants as long as individual molecules can be identified. The starting point of this method is a correlation function, analogous to spatiotemporal image correlation spectroscopy (STICS) (138, 191). A qualitative criterion for the general applicability is given. Further, theoretical boundaries for the achievable accuracy are discussed. Finally, the validity of the method is demonstrated by application to data created by Monte Carlo simulations and analysis of experimental data (131). The latter proves the existence of functional domains smaller than 200 nm in the plasma membrane of 3T3-A14 fibroblast cells.

5.2 Theory

For clarity, we develop the method for the ideal situation, without e.g. bleaching of molecules. In App. 5.A a rigorous treatment of non-ideal situations is given which includes the effects of a limited field of view, finite positional accuracy, finite exposure time, bleaching and blinking of molecules.

5.2.1 Algorithm

An image I obtained from SPT experiments is described as a sum of delta peaks which represent the positions $\mathbf{r}_i$ of the molecules:

$$I(\mathbf{r}) = \sum_{i=1}^m \delta(\mathbf{r} - \mathbf{r}_i) , \quad \mathbf{r} = (x, y) \quad (5.1)$$

Here m is the number of molecules in image I . The delta functions represent only the positions of the molecules and therefore information about the intensity of the molecules is discarded in Eq. 5.1. The positions are retrieved from the raw image by fitting with the point spread function of the microscope as detailed in (114). For any pair of images, I_a and I_b , which are separated in time by a time lag of Δt , a spatiotemporal

correlation function is defined:

$$C(\mathbf{d}, \Delta t) = \frac{\langle \iint_A d\mathbf{r} I_a(\mathbf{r}) I_b(\mathbf{r} + \mathbf{d}) \rangle_{\Delta t}}{\langle m_a \rangle} \quad (5.2)$$

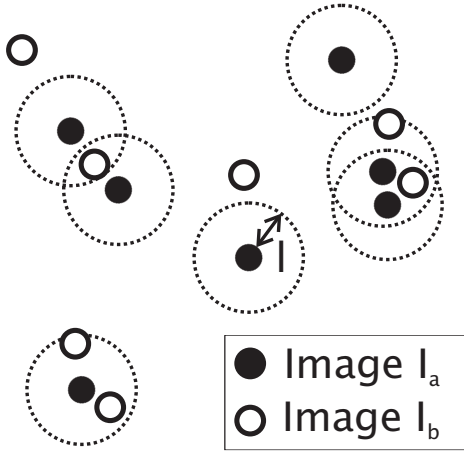
where $\langle \dots \rangle_{\Delta t}$ denotes the ensemble average over all pairs of images separated by a time lag Δt and A is the area of the field of view of the microscope. The two images are shifted by $\mathbf{d}$ with respect to each other and subsequently correlated, i.e. the spatial integral of their product is calculated. If $\mathbf{d}$ coincides with a movement during the time-lag Δt the correlation will be high. The precise connection to the diffusion dynamics will be given below. Note that $C(\mathbf{d}, \Delta t)$ is basically the correlation function used in STICS (138, 191) where here the denominator is given by the average number of molecules in image I_a only. This normalization was chosen since it leads directly to the cumulative probability distribution of diffusion steps, see Eq. 5.5.

In an isotropic medium the cumulative correlation function only depends on a distance l and time-lag Δt . By definition of $\mathbf{d}(\rho, \phi) = (\rho \cdot \cos \phi, \rho \cdot \sin \phi)$ with polar coordinates ρ and ϕ

$$\begin{aligned} C_{\text{cum}}(l, \Delta t) &= \int_0^l d\rho \rho \int_0^{2\pi} d\phi C(\mathbf{d}(\rho, \phi), \Delta t) \\ &= \frac{\langle \iint_A d\mathbf{r} I_a(\mathbf{r}) m_b(\mathbf{r}, l) \rangle_{\Delta t}}{\langle m_a \rangle} \\ &= \frac{\langle \sum_{i=1}^{m_a} m_b(\mathbf{r}_{ai}, l) \rangle_{\Delta t}}{\langle m_a \rangle} \end{aligned} \quad (5.3)$$

where $\mathbf{r}_{ai}$ is the position of molecule i in image I_a and $m_b(\mathbf{r}, l) = \int_0^l d\rho \rho \int_0^{2\pi} d\phi I_b(\mathbf{r} + \mathbf{d}(\rho, \phi))$. $m_b(\mathbf{r}, l)$ is the number of molecules in image I_b that lie in a circle with radius l around $\mathbf{r}$.

The algorithm to obtain $C_{\text{cum}}(l, \Delta t)$ from experimental data, derived directly from Eq. 5.3 and the definition of $m_b(\mathbf{r}, l)$, is illustrated in Fig. 5.1: for each molecule position $\mathbf{r}_{ai}$ in image I_a the number of molecules in image I_b are counted whose distance to $\mathbf{r}_{ai}$ is smaller or equal to l . Subsequently the contributions from all molecules in image I_a are summed and averaged over all image pairs. The division by the average number of molecules in image I_a finally results in $C_{\text{cum}}(l, \Delta t)$.

**Figure 5.1**

Particle image correlation spectroscopy algorithm. For each molecule in image I_a (open circles) the number of molecules in image I_b (closed circles) closer than l is counted (5 in this example). Note that the peak in the center that lies within the overlap of two circles will be counted twice. Hence, the contribution that is due to diffusion is 4 whereas 1 count is due to random spatial proximity of molecules.

5.2.2 Relation to diffusion dynamics

$C_{\text{cum}}(l, \Delta t)$ contains both temporal (i.e. diffusion of molecules) and spatial (i.e. random spatial proximity of molecules) correlations which will be separated below. The spatial correlations are illustrated by the overlap of the circles in Fig. 5.1. Given that the molecules are identical, their movement is mutually uncorrelated, and the medium is homogeneous, $C_{\text{cum}}(l, \Delta t)$ is simplified to

$$C_{\text{cum}}(l, \Delta t) = \langle m_b(\tilde{\mathbf{r}}, l) \rangle_{\Delta t} \quad (5.4)$$

where $\tilde{\mathbf{r}}$ is the arbitrary position of a molecule in image I_a . Note that the summation in Eq. 5.3 cancels out with the denominator $\langle m_a \rangle$ under the given assumptions. It should be mentioned that a global flow of the molecules is admissible. The same holds for interactions between molecules if they can be sufficiently described by a mean-field approximation. The part of Eq. 5.4 that is caused by accidental spatial proximity of different molecules is equal to the mean number of molecules in a circle with radius l around a certain fixed but arbitrary molecule. Given that the molecules are distributed uniformly and independently with a density c the probability to find μ molecules in this circle is given by a Poisson distribution with mean and variance of: $\bar{\mu} = (\mu - \bar{\mu})^2 = c \cdot \pi l^2$, where c can be estimated as $c = (\langle m_b \rangle - 1)/A$. The latter assumption is justified given that the ensemble average usually comprises many images of many different cells. Note that the precise definition of c is the density of the

neighbors of a certain molecule. For higher densities this equals the total density since then $(\langle m_b \rangle - 1)/A \approx \langle m_b \rangle/A$.

The part of Eq. 5.4 that contains the diffusion dynamics of the molecules is equal to the cumulative probability $P_{\text{cum}}(l, \Delta t)$ to find a diffusion step with a size smaller than l if the time-lag is Δt . For normal diffusion with diffusion coefficient D in two dimensions $P_{\text{cum}}(l, \Delta t) = 1 - \exp\left(-\frac{l^2}{4D\Delta t}\right)$

The combination of both contributions leads to the following form of $C_{\text{cum}}(l, \Delta t)$:

$$C_{\text{cum}}(l, \Delta t) = P_{\text{cum}}(l, \Delta t) + c \cdot \pi l^2 \quad (5.5)$$

The quantity calculated from experimental data by the algorithm described above (Eq. 5.3) is an estimator for this theoretically expected value. We now define a typical lengthscale l_{cum} by

$$P_{\text{cum}}(l_{\text{cum}}, \Delta t) = 1/2 \quad (5.6)$$

. After subtraction of $c \cdot \pi l^2$ from C_{cum} this length scale can be determined and the diffusion constant is then calculated as:

$$D\Delta t = \frac{1}{\ln 2} \cdot \left(\frac{l_{\text{cum}}}{2}\right)^2 \quad (5.7)$$

5.2.3 Figure of merit and achievable accuracy

Determination of $P_{\text{cum}}(l, \Delta t)$ from Eq. 5.5 is only practical if the variance of the second term $c \cdot \pi l^2$ is sufficiently small, as detailed below. Since the average of M statistically uncorrelated pairs of images is taken the variance is $1/M$ times the value given above for the single Poisson process. Note that successive *pairs* of images are statistically uncorrelated since diffusion is a Markov process, whereas successive images are necessarily correlated. In order to get a qualitative criterion for the number of image pairs to be taken for a significant result the standard deviation of the spatial correlations at l_{cum} (given by Eq. 5.7) is compared to the value of $P_{\text{cum}}(l, \Delta t)$ at l_{cum} :

$$\sqrt{\frac{c\pi l_{\text{cum}}^2}{M}} \ll \frac{1}{2} \quad (5.8)$$

We define a figure of merit η as twice this standard deviation

$$\eta = \sqrt{16\pi \ln 2 \cdot \frac{c \cdot D\Delta t}{M}} \quad (5.9)$$

Thus the result will be significant if $\eta \ll 1$. Note that molecules may be arbitrarily dense (provided that the overlapping images still allow them to be identified as individual molecules) or diffuse arbitrarily fast if only the number M of image-pairs is sufficiently large.

If the whole correction term is small, $c \cdot \pi l^2 \ll 1$, i.e.

$$8\pi \ln 2 \cdot c \cdot D\Delta t \ll 1 \quad (5.10)$$

we directly obtain

$$C_{\text{cum}}(l, \Delta t) \approx P_{\text{cum}}(l, \Delta t) \quad (5.11)$$

To get an error estimate for the diffusion constant D the probability density $P_{\text{cum}}(l, \Delta t)$ is shifted vertically by $\pm \eta/2$. From the calculation of the typical length scale l_{cum} of the shifted curves, boundaries for the values of D are retrieved:

$$\frac{1}{2} = P_{\text{cum}}(l_{\text{cum}}, \Delta t) \pm \frac{\eta}{2} \Rightarrow \frac{\Delta D}{\bar{D}} \approx \frac{\eta}{\ln 2} \quad (5.12)$$

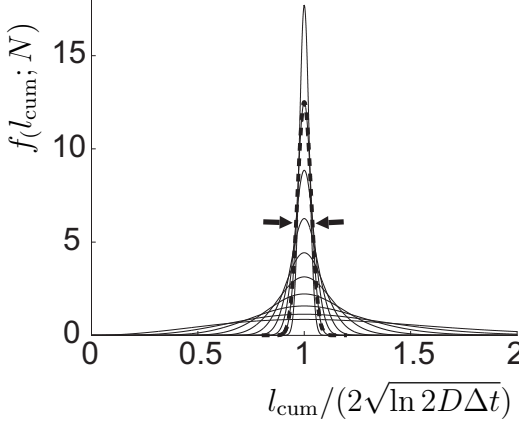
for a sufficiently small η . $\bar{D}$ designates the mean D .

While this error originates from the method, there is an intrinsic spread of the values obtained for l_{cum} that is due to the stochastic nature of diffusion. If M pairs of images with $\langle m \rangle$ molecules on the average are acquired the number of diffusion steps to be analyzed is $N = M \cdot \langle m \rangle$. The probability to find $N/2$ steps with a step-size smaller than l_{cum} is given by

$$f(l_{\text{cum}}; N) = K \cdot P_{\text{cum}}(l_{\text{cum}}, \Delta t)^{\frac{N}{2}} (1 - P_{\text{cum}}(l_{\text{cum}}, \Delta t))^{\frac{N}{2}} \quad (5.13)$$

where K is a normalization factor determined by $\int_0^\infty dl_{\text{cum}} f(l_{\text{cum}}; N) = 1$. This probability density for l_{cum} is depicted in Fig. 5.2 for various values of N .

For an increasing number of diffusion steps, N , the function becomes symmetric about the value given by Eq. 5.7 and the width decreases. Hence the more images analyzed the less the spread in l_{cum} . Expansion of the exponentials in Eq. 5.13 around the maximum and estimation of the relative width for $N \gg 1$ yields $\Delta l_{\text{cum}}/\bar{l}_{\text{cum}} = (1/(2 \ln 2)) \sqrt{1 - (1/2)^{2/N}}$ where $\bar{l}_{\text{cum}}$ designates the mean l_{cum} and equals the value given by Eq. 5.7. Note that Δl_{cum} is defined analogous to the standard deviation as half the width of Eq. 5.13. Error propagation gives $\Delta D/\bar{D} = 2 \cdot \Delta l/\bar{l}_{\text{cum}}$. To determine D with a relative error of ± 0.1 , $N \approx 300$ diffusion steps are

**Figure 5.2**

Probability density $f(l_{\text{cum}}; N)$ versus $l_{\text{cum}}/(2\sqrt{\ln 2D\Delta t})$ for $N = 2, 4, 8, \dots, 1024$. The curve for $N = 1024$ corresponds to the sharpest distribution. For $N = 512$ (dashed curve) expansion around the maximum was used to estimate the width of the distribution. Arrows indicate $2 \cdot (\Delta l_{\text{cum}}/(2\sqrt{\ln 2D\Delta t}))$.

needed. Since the accuracy scales as $1/\sqrt{N}$ for $N \gg 1$ a relative error of ± 0.01 requires $N \approx 30000$ steps. Note that this error estimation is only valid if the diffusion coefficient is determined from the typical length scale l_{cum} of $P_{\text{cum}}(l, \Delta t)$. For the scatter inherent to other analysis methods see the paper by Saxton (137).

Since the described errors are uncorrelated the total error is

$$\frac{\Delta D_{\text{total}}}{D} = \frac{1}{\ln 2} \sqrt{\eta^2 + (1 - (1/2)^{2/N})} \quad (5.14)$$

For the adaptation of the method to non-ideal situations that include e.g. bleaching see App. 5.A.

5.2.4 Diffusion modes

Given that the criterion below Eq. 5.9 is fulfilled the method developed up to this point is exact for the case of a single, normally diffusing species. For other (anomalous) cases (multiple fractions, intermittent, confined or anisotropic diffusion, diffusion with trapping or, more generally, diffusion in a potential landscape) the diffusion coefficient determined as described in Sec. 5.2.2 is only an estimation of the mean diffusion coefficient.

However, since the cumulative probability of step-sizes is intrinsic to the correlation function Eq. 5.5, analysis of data with more complicated diffusion models is straightforward. E.g. for a two-fraction case, which is important for the data analyzed below, molecules in image I_a are split in a fraction of size α with diffusion coefficient D_1 and one of size $1 - \alpha$ with diffusion coefficient D_2 . This results in:

$$P_{\text{cum}}(l, \Delta t) = \alpha \left(1 - \exp \left(-\frac{l^2}{r_1^2} \right) \right) + (1 - \alpha) \left(1 - \exp \left(-\frac{l^2}{r_2^2} \right) \right) \quad (5.15)$$

where $r_i^2 = 4D_i\Delta t$, $i \in \{1, 2\}$. Hence, the probability distribution $P_{\text{cum}}(l, \Delta t)$ can faithfully be used to analyze more complex inhomogeneous diffusion behavior.

5.3 Materials and Methods

5.3.1 Monte Carlo simulations

For validation of the method a Monte Carlo approach was used to generate random diffusion steps and determine the diffusion coefficient as described in Sec. 5.2.1. All simulations were performed within the *Matlab* programming environment. With the help of the standard *Matlab* routines for random number generation M pairs of images were generated in the following way: the first image I_a consists of molecule signals scattered uniformly over an area A_{sim} which was bigger than the physical field of view of area A . This was necessary for the simulation of molecules that enter the area A during Δt . A_{sim} was taken large enough for the distribution of the molecules to be still approximately uniform in A after each time step Δt . The average number of molecules in A was fixed at 5. Image I_b was obtained by letting each molecule in I_a perform a random step in x and y direction. The step-size in both spatial directions was determined by a Gaussian with variance $2D\Delta t$, i.e. all simulated molecules obeyed normal diffusion. Subsequently, all molecules that did not fall into the physical field of view were discarded. Furthermore it was ensured that diffusion steps up to l_{max} were adequately represented as detailed in App. 5.A. The algorithm derived in Sec. 5.2.1 was subsequently executed for the values $l = \delta l, 2 \cdot \delta l, \dots, l_{\text{max}}$.

l_{cum} was found from $P_{\text{cum}}(l, \Delta t)$ by linear interpolation of the distribution around 0.5. The results were normalized to $2\sqrt{\ln 2 D\Delta t}$ such that, according to Eq. 5.7, a value of 1 corresponds to the most probable l_{cum} . The whole simulation was repeated 1000 times and the results were divided into bins of width 0.05. The number of data points in each bin was subsequently divided by 1000 which resulted in relative frequencies for l_{cum} . For comparison of the simulation with theoretical predictions, the probability density derived in Eq. 5.13 was integrated over intervals of length 0.05, i.e. the bin size.

Since only a finite amount of values for l can be considered, a binning error, that depends on δl is introduced. Consequently, the distribution of the l_{cum} values will always deviate from Eq. 5.13. In Fig. 5.3, results

for $\delta l = 0.01 \cdot \sqrt{D\Delta t}$, $0.5 \cdot \sqrt{D\Delta t}$ and $\sqrt{D\Delta t}$ are compared with $l_{\max} = 3$. Since we choose a very small density and diffusion coefficient ($c = 2.5 \cdot 10^{-4}/\mu\text{m}^2$, $D\Delta t = 0.02\mu\text{m}^2$), the deviation from the theoretical distribution Eq. 5.13 is caused by the binning error alone. Obviously the deviation decreases with decreasing δl . The simulations therefore use $\delta l = 0.01 \cdot \sqrt{D\Delta t}$. For smaller or bigger diffusion coefficients or time-lags, $l_{\max}$ is scaled accordingly.

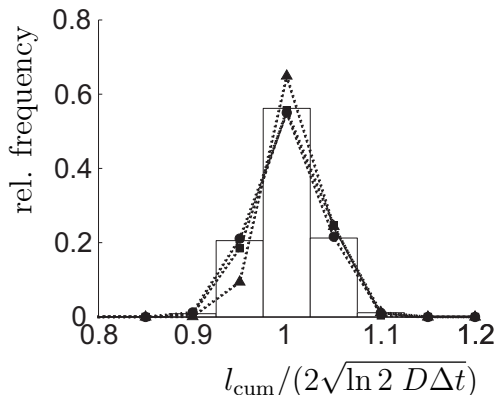


Figure 5.3

Binning error introduced into the estimation of l_{cum} . 100 image pairs with diffusion constant $D = 1\mu\text{m}^2/\text{s}$, $\Delta t = 20\text{ms}$ at a concentration of $c = 2.5 \cdot 10^{-4}/\mu\text{m}^2$ were used. The binning was set to triangle: $\delta l = \sqrt{D\Delta t}$; square: $\delta l = 0.5 \cdot \sqrt{D\Delta t}$; circle: $\delta l = 0.01 \cdot \sqrt{D\Delta t}$, and compared to the distribution as given by Eq. 5.13 with $N = 500$ (bars).

5.3.2 Single-molecule microscopy

The experiments were described in detail previously (131). In short constitutive active human H-Ras (V12) and constitutive inactive human H-Ras (N17) were coded into pcDNA3.1-eYFP (Qiagen, Hilden, Germany). Cells from a mouse fibroblast cell line stably expressing the human insulin receptor (3T3-A14) (192) were transfected with $1.0\mu\text{g}$ DNA and $3\mu\text{l}$ FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, USA) per glass slide. 3T3-A14 cells adhered to glass slides were mounted onto the microscope and kept in PBS at 37°C . For the observation of the mobility of individual eYFP-H-Ras molecules the focus of the microscope was set to the dorsal surface membrane of individual cells (depth of focus $\approx 1\mu\text{m}$). The density of fluorescent proteins on the plasma membrane of selected transfected cells was less than $1\mu\text{m}^{-2}$ to permit imaging and tracking of individual fluorophores. Molecule positions were determined with an accuracy of $\approx 35\text{nm}$. Fluorescence images were taken consecutively with up to 1000 images per sequence. Typical trajectories were up to 9 steps in length, mainly limited by the blinking and photobleaching of the fluorophore (110). Data sets were acquired with different time lags Δt between

consecutive images. Δt varied from 5 to 60 ms.

5.4 Results

5.4.1 Monte Carlo simulations

The influence of a growing molecule density, c , and number of acquired image pairs M on the distribution were investigated for fixed $D\Delta t$. The simulated concentrations correspond to a range of $0.1 - 10$ molecules/ μm^2 for typical experimental values ($D \approx 1 \mu\text{m}^2/\text{s}$, $\Delta t \approx 20 \text{ ms}$).

The results for $M = 100$ and $M = 1000$ are presented in Fig. 5.4.

For fixed M the distribution of l_{cum} values broadens with rising molecule concentration. It should be noted that the distribution of l_{cum} always peaked around the true value. When the correction term for correlations due to random spatial proximity of molecules was omitted (i.e. the second term in Eq. 5.5) the peak l_{cum} values shifted to a lower value. Likewise the dependence of the method on the diffusion constant D and the number of image pairs M was studied for a fixed molecule density. For typical experimental values ($c \approx 1/\mu\text{m}^2$, $\Delta t \approx 20 \text{ ms}$) the diffusion constants correspond to a range from $0.1 \mu\text{m}^2/\text{s}$ to $10 \mu\text{m}^2/\text{s}$. Results are shown in Fig. 5.4. The distribution broadens with D similar to the results for growing molecule density. As predicted by Eq. 5.12, the distributions become narrower for growing M which supports the claim that a higher number of image-pairs will compensate for a high molecular density or diffusion constant. The applicability of the method is, therefore, only limited by the amount of images that can be acquired for identical conditions. The influence of bleaching and blinking on the distribution of l_{cum} is shown in Fig. 5.5.

Molecules were assumed to turn dark with a probability p_{dark} per time-lag Δt . The distribution broadens if this probability is increased but stays peaked around the true value. The broadening is fully accounted for by the reduction of the statistical sample size $N = M\langle m \rangle$. E.g. for $p_{\text{dark}} = 0.9$ only 10% molecules survive leaving only 50 visible diffusion steps instead of 500 for $p_{\text{dark}} = 0$. We do not consider explicitly here that molecules can return into the fluorescent state (blinking) since the only effect is an increase in the apparent molecule density c , which was analyzed above.

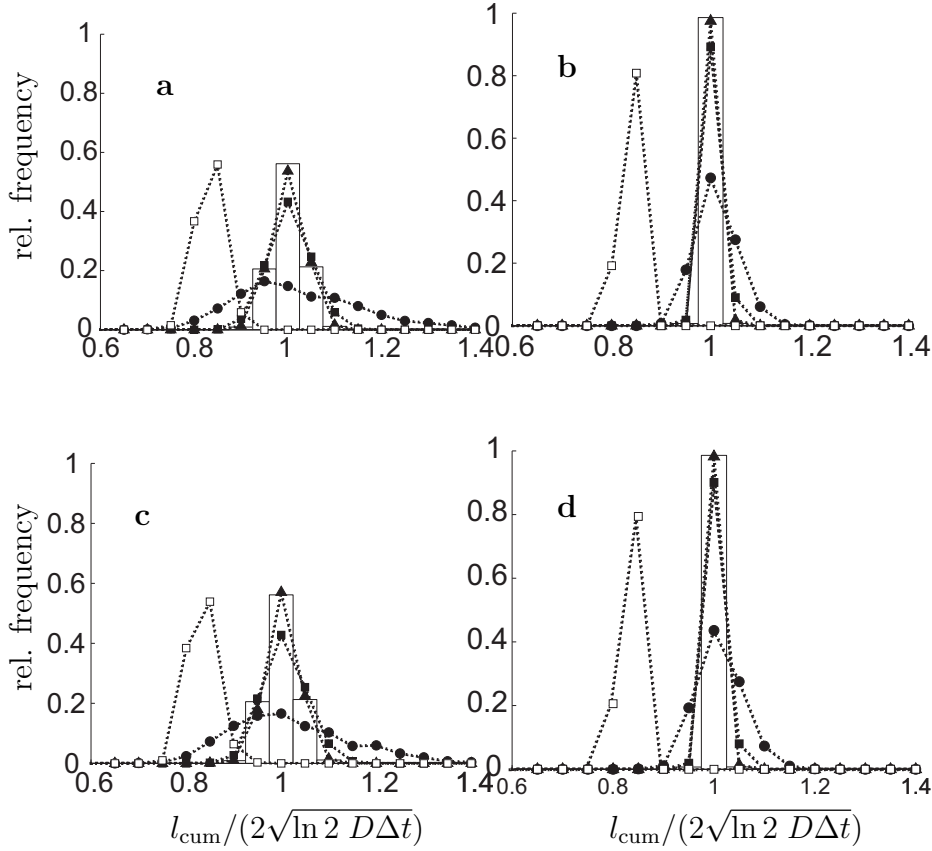


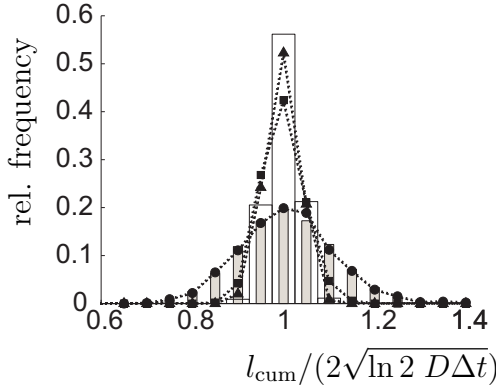
Figure 5.4

Distribution of l_{cum} from simulations. (a & b) Influence of molecule concentration at fixed $D\Delta t = 1 \mu\text{m}^2/\text{s}$ and given number of images $M = 100$ (a), and $M = 1000$ (b) (solid triangle : $c = 0.1/\mu\text{m}^2$; solid square: $c = 1/\mu\text{m}^2$; solid circle: $c = 10/\mu\text{m}^2$; open square: same values as for the solid squares but without correction term; bars: distribution as given by Eq. 5.13 with $N = 500$ (a) and $N = 5000$ (b)).

(c & d) Influence of rising diffusion constant for constant $c = 1/\mu\text{m}^2$ and given number of images $M = 100$ (c), and $M = 1000$ (d) (solid triangle : $D\Delta t = 0.1 \mu\text{m}^2/\text{s}$; solid square: $D\Delta t = 1 \mu\text{m}^2/\text{s}$; solid circle: $D\Delta t = 10 \mu\text{m}^2/\text{s}$; open square: same values as for the solid squares but without correction term; bars: distribution as given by Eq. 5.13) with $N = 500$ (c) and $N = 5000$ (d)).

5.4.2 Diffusional behavior of H-Ras mutants

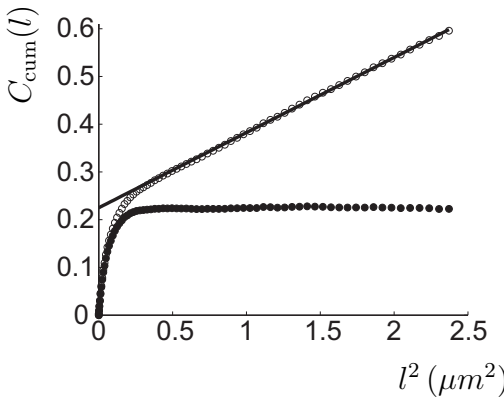
Following the simulations, data on tracking individual H-Ras mutants on the plasma membrane of 3T3-A14 cells at 37°C , was analyzed. In a pub-

**Figure 5.5**

Distribution of l_{cum} from simulations including photobleaching. 100 image pairs were analyzed at a concentration of $0.1/\mu\text{m}^2$, diffusion constant $D = 1\mu\text{m}^2/\text{s}$ and time-lag $\Delta t = 20\text{ms}$ (triangle : $p_{\text{dark}} = 0$; square : $p_{\text{dark}} = 0.4$; circle : $p_{\text{dark}} = 0.9$; white bars: distribution as given by Eq. 5.13 with $N = 500$; gray bars: distribution as given by Eq. 5.13 with $N = 50$).

lication by Lommerse et al. (131) it was found that both the constitutive inactive (N17) as well the constitutive active (V12) variant of the protein displayed an inhomogeneous two-fraction diffusion behavior. In that earlier report the positions of proteins in an image sequence were used to calculate trajectories from which further information on the mobility was extracted. Here the same position data is analyzed with the new algorithm without any *a priori* knowledge about molecular mobility.

The molecule density c was estimated from the experimental data. The slope of the linear part of $C_{\text{cum}}(l)$ when plotted versus l^2 (Fig. 5.6) directly equals $c \cdot \pi$. Note that c is by definition of this procedure exactly the density of neighboring molecules introduced in Sec. 5.2.2. Subtraction of

**Figure 5.6**

Experimentally obtained cumulative correlation function $C_{\text{cum}}(l)$. $C_{\text{cum}}(l)$ was obtained for individual H-Ras(N17) molecules at the apical side of 3T3-A14 cells with a time-lag Δt of 20ms (open circles: $C_{\text{cum}}(l)$; solid line: linear fit of the long distance data yielded a concentration $c \approx 0.16/\mu\text{m}^2$; closed circle: after subtraction of the correction term). Fit of the corrected data to Eq. 5.15 yielded $\alpha = 0.90 \pm 0.02$, $r_1^2 = 0.072 \pm 0.002\mu\text{m}^2$, and $r_2^2 = 0.012 \pm 0.0003\mu\text{m}^2$.

the correction term $c \cdot \pi l^2$ successfully yielded $P_{\text{cum}}(l, \Delta t)$ for longer time-lags (solid data points in Fig. 5.6). Artifacts due to diffraction observed for

shorter time-lags, were removed by an empirical, self-consistent algorithm as detailed in Appendix 5.B.

$P_{cum}(l, \Delta t)$ was subsequently constructed for each time lag Δt between 4 and 60 ms. Data were fit according to the two diffusing fractions model (Eq. 5.15) to yield the fraction α and respective mean square displacements r_1^2 and r_2^2 for both mutants.

Fig. 5.7 compares the results obtained by the new unbiased method (full symbols, solid lines) with those obtained by conventional tracking methods (open symbols, dashed lines) in which an initial diffusion constant of $D = 1\mu m^2/s$ had been assumed.

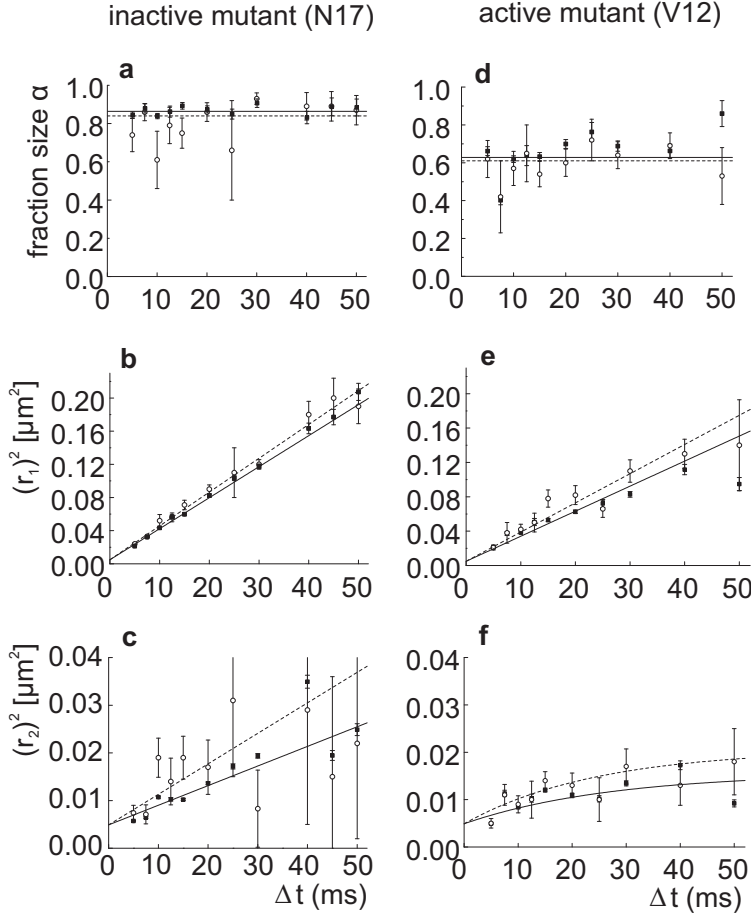
Both data sets excellently match each other within experimental accuracy, see Tab. 5.1.

	Tracking	PICS
HRas(N17)		
$D_1(\mu m^2/s)$	1.02 ± 0.02	0.94 ± 0.01
$D_2(\mu m^2/s)$	0.16 ± 0.03	0.10 ± 0.01
α	0.84 ± 0.05	0.86 ± 0.01
HRas(V12)		
$D_1(\mu m^2/s)$	0.85 ± 0.04	0.73 ± 0.01
$D_2(\mu m^2/s)$	0.16 ± 0.04	0.10 ± 0.01
$L(nm)$	217 ± 46	179 ± 10
α	0.61 ± 0.05	0.63 ± 0.01

Table 5.1

Comparison between results obtained by conventional tracking with results obtained by PICS.

For the inactive mutant (N17) 86% of the molecules fell into the highly mobile fraction characterized by a diffusion constant of $D_1 = 0.94\mu m^2/s$. The slow fraction was characterized by a diffusion constant of $D_2 = 0.10\mu m^2/s$. Both fractions followed free diffusion as seen by the linear dependence of the mean square displacements (r_i^2) with Δt . In contrast, the slow diffusing fraction of the active mutant (V12) displayed a confined diffusion behavior (29) characterized by a confinement size of $L = 179nm$. In addition, the diffusion constant of the fast, free diffusion fraction of the V12-mutant was reduced to $D_1 = 0.73\mu m^2/s$ and the fraction size decreased to 63% in comparison to the inactive mutant N17.

**Figure 5.7**

Diffusional behavior of HRas. Fraction α (a & d) and mean square displacements r_1^2 (b & e) and r_2^2 (c & f) as functions of Δt for the constitutive inactive (N17) (a-c) and the constitutive active (V12) mutant (d-f) of HRas. Open circles / dashed lines correspond to conventional tracking results (131); solid squares / solid lines to results obtained by the PICS method. In the case of the conventional tracking errorbars correspond to the error of the fitting of the two fraction model, for PICS the size of the errorbars is given by Eq. 5.14.

5.5 Discussion

The combination of the advantages of two well-established techniques, ICM and SPT, allowed the development of a robust analysis method which retrieves spatiotemporal correlations on the sub-wavelength and millisecond time scale. By Monte-Carlo simulations the principle was proven and it was shown that the method can deal with short traces, high molecule densities and high diffusion constants provided that individual molecules can be identified and the total number of diffusion steps is sufficiently high. This holds even without an initial guess of the diffusion coefficients. Application to real experimental data shows that the method is simpler than conventional tracking while identical results are obtained. Structures with a diameter of $< 200\text{ nm}$ were faithfully identified. It should be noted however, that the method is not applicable for non-ergodic systems, i.e. if it becomes important that different molecules have different spatial environments. If the movement of the molecules is highly correlated, e.g. for interactions which cannot be handled by a mean-field approach, correction schemes like the one presented in the Appendix have to be employed.

The results of change in mobility on the activation state of HRas by the new unbiased method further supports ideas of functional domains in the plasma membrane of mammalian cells. The results agree well with the results of the FRET (93), FRAP (193), EM (194) and single-molecule tracking experiments (131) in all of which functional domains have been observed. Likely localization of active HRas to these functional domains is not a static process, but is dynamic as suggested for trapping into cholesterol-independent domains (194) and into more general transient signaling complexes (93) which might be actin dependent.

In summary, a robust method was presented that is superior to both ICM and SPT surpassing the first in resolution and largely simplifying the analysis methods required for the second. Another intriguing application is the study of dynamical properties of interacting proteins in model membranes. Because the newly developed method allows the protein concentration to be varied over a wider range, a comparison to theoretical results obtained by a virial expansion is rendered possible.

5.A Beyond the ideal situation

Limited field of view In the experimental situation the field of view is always limited. Typically in the case of an epi-fluorescence setup the field of view is chosen in the center of the Gaussian beam profile so that the illumination can be considered uniform. Molecules which diffuse out of view not only limit the observation time but it is also more probable for a long step to end out of the field of view than for a small step. Consequently long steps are under-represented in the experimental distribution. Therefore a reduced field of view is defined which has a width that is smaller than the full field of view by an amount of $2 \cdot l_{\max}$. Only those peaks of image I_a that lie within the reduced field of view are used. Thus, no steps are lost up to a length of $l_{\max}$.

Finite positional accuracy The limited positional accuracy makes a fixed molecule appear to move and a free molecule to diffuse faster. Since the real diffusive motion and the apparent motion due to the limited positional accuracy are uncorrelated, the fluctuations simply add so that

$$D_{\text{meas}}\Delta t = D_{\text{real}}\Delta t + \sigma^2 \quad (5.16)$$

where D_{meas} is the measured diffusion coefficient, D_{real} is the real diffusion coefficient and σ is the standard deviation of a Gaussian distribution that describes the positional error in one dimension. Either the positional accuracy has to be determined independently or the time lag Δt must be varied so that the real diffusion coefficient can be obtained from the slope of Eq. 5.16. Note that this problem does not interfere with the method presented here; e.g. in the case of normal diffusion of one or two molecular species the functional form of the cumulative probability distribution P_{cum} remains unchanged. For other diffusion modes the correct form of P_{cum} , which might be altered due the finite positional accuracy, has to be employed. An extensive discussion can be found in (195).

Finite exposure / frame integration time The fact that the fluorescence signal collection and integration time is finite can lead to erroneous results, in particular for confined diffusion (35, 196). However, it was shown in (196) that the true values for the diffusion coefficient and the size of the confinement area can be retrieved from the data anyway. For the analysis performed in Sec. 5.4.2 we assume that the influence of

confinement or a finite exposure time on the cumulative probability distribution $P_{\text{cum}}(l, \Delta t)$ is negligible compared to the experimental error. This is quantified by the criterion given in (196): if L is the linear size of the confinement, D is the diffusion coefficient and T is the exposure / integration time then $T \ll L^2/12D$ should be fulfilled. This is indeed the case for the experiments presented in Sec. 5.4.2 with $L \approx 0.18 \mu\text{m}$, $D = 0.1 \mu\text{m}^2/\text{s}$ and $T = 3\text{ms}$. So, it is sensible to expect a distribution representing normal diffusion. It should, however, be stressed that our method works in principle for arbitrary forms of $P_{\text{cum}}(l, \Delta t)$.

Bleaching and blinking Because of blinking and bleaching, single-particle trajectories of biologically relevant fluorophores inside cells are usually short (≈ 10 steps). Given that p_{off} is the probability per time-lag Δt that a molecule turns dark or is not found by the peak fitting algorithm (see also Appendix 5.B) only a fraction $(1 - p_{\text{off}})$ of all diffusion steps is observed. Under the assumption that bleaching is independent of the size of a diffusion step, P_{cum} is reduced by a factor $(1 - p_{\text{off}})$. One consequence is that the figure of merit (Eq. 5.9) must be generalized to:

$$\eta = \frac{1}{(1 - p_{\text{off}})} \sqrt{16\pi \ln 2 \cdot \frac{c \cdot D \Delta t}{M}} \quad (5.17)$$

Accordingly Eq. 5.10 changes to:

$$8\pi \ln 2 \cdot \frac{c \cdot D \Delta t}{(1 - p_{\text{off}})} \ll 1 \quad (5.18)$$

The second consequence is that the experimental correlation function C_{cum} has to be normalized to 1, after subtraction of the correction term $c\pi l^2$, to yield P_{cum} (see also Appendix 5.B). Correspondingly, the theoretical distribution function has to be divided by $P_{\text{cum}}(l_{\text{max}}, \Delta t)$ where l_{max} is the maximal l included in the analysis.

5.B Correction for positional correlations due to diffraction

Due to diffraction, the imaged Airy disks of the fluorescent molecules have a finite width and two molecules separated by a distance smaller than this width cannot be resolved. Therefore, one or both molecules will be absent

in the position data. Consequently, fewer molecules are found close to each other than expected from the average molecule density. Thus, the molecule positions that ultimately enter into the analysis are effectively correlated. In the cumulative correlation function C_{cum} , determined from experimental data, this is visible as a dip for small step-sizes, see Fig. 5.8.

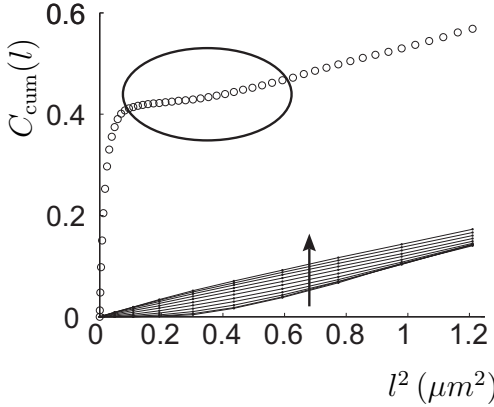


Figure 5.8

Correction for random spatial proximity of molecules at short distances and short time-lag. The dip in the data obtained for individual H-Ras(N17) molecules at the apical side of 3T3-A14 cells taken at a time delay of 5ms is due to diffraction (open circles: raw data; solid lines: pure spatial correlation for distances r from an arbitrary molecule, $r = 0 \mu\text{m}, 0.11 \mu\text{m}, 0.22 \mu\text{m}, \dots, 1.21 \mu\text{m}$ where r rises in the direction of the arrow).

Since the correlation length is of the order of the peak width ($\approx 0.4 \mu\text{m}$) this effect is only observable for small step-sizes, i.e. for slowly diffusing molecules or small time-lags. To circumvent this problem we adapted our algorithm in the following way: in the simple estimation the number of “wrong” connections that the algorithm makes is described by the quadratic correction term $c \cdot \pi l^2$; now the amount of molecules that are found within a certain radius depends on the size of the diffusive step. If the molecule turns dark during the time-lag there is no correlation. Therefore Eq. 5.5 is generalized to

$$C_{\text{cum}}(l, \Delta t) = (1 - p_{\text{off}})P_{\text{cum}}(l, \Delta t) + p_{\text{dark}}c \cdot \pi l^2 + (1 - p_{\text{dark}}) \int_0^\infty dr s(r, l) \frac{\partial P_{\text{cum}}}{\partial r}(r, \Delta t) \quad (5.19)$$

where the function $s(r, l)$ gives the number of molecules in a circle with radius l if the diffusive step-size is r . $\partial P_{\text{cum}}(r, \Delta t)/\partial r$ gives the probability for a step of length r . p_{dark} , the probability per time-lag that a molecule turns dark, is estimated once and kept fixed for all data sets. For the data analyzed above $p_{\text{dark}} = 0.3$ was used. p_{off} is the probability that a molecule either turns dark or is not found by the molecule fitting routine

e.g. since it came too close to another molecule. $1 - p_{\text{off}}$ can be estimated by the height of C_{cum} after subtraction of the correction term. $s(r, l)$ is determined empirically from the experimental data by application of the algorithm defined in the beginning where, however, images I_a and I_b are identical. Furthermore the center of the circle, with radius l , in which the molecules are counted is translated by a vector of length r in arbitrary direction. The average over 20 equally spaced directions results in the array of curves depicted in Fig. 5.8. Subsequent to the calculation of $s(r, l)$ the correction is determined numerically by the following self-consistent algorithm:

1. as an initial guess for the correction term determine the slope of the linear part of C_{cum} and use the original correction term from Eq. 5.5.
2. subtract the correction.
3. normalize to 1 and fit the model.
4. calculate the new correction according to Eq. 5.19, go to step 2.

Steps 2 to 4 are repeated until the fit parameters change less than a predefined threshold. Note that this approach to correct for the effective correlation of the peak positions only works because the effect is the same for all molecules. If positional correlations that are different for different molecules become important the approach is no longer functional.

CHAPTER 6

ROLE OF MEMBRANE HETEROGENEITY AND PRECOUPLING IN ADENOSINE A_1 RECEPTOR SIGNALING UNRAVELED BY PARTICLE IMAGE CORRELATION SPECTROSCOPY (PICS)

We use the recently developed particle image correlation spectroscopy (PICS) to unravel the influence of spatial membrane organization on adenosine A_1 receptor signaling. It turns out that the diffusion behavior of the receptor can be used as a faithful readout of its activation state. We identify slowed diffusion with localization of the receptor in membrane microdomains. Stimulation experiments show that 9 % of the receptors translocate to ≈ 130 nm sized domains upon agonist exposure. In experiments on cell blebs this structure is lost. This shows that the observed domains are closely linked with the cytoskeleton. Decoupling of receptor and G protein leads to an increase in receptor mobility in 7 % of the cases. This we take as evidence for receptor G-protein precoupling corroborating that the G protein is responsible for the interaction with microdomains.

6.1 Introduction

The adenosine A₁ receptor is involved in many physiological processes ranging from neuroprotective mechanisms in the brain (197) to the control of heart rate (144). It belongs to the superfamily of G-protein coupled receptors (GPCRs), of which many fulfill clinically significant functions. For this reason, and because many GPCRs are conveniently localized in the cell's plasma membrane, they are attractive drug targets (144). The wish to create more effective and specific drugs motivates the interest in understanding GPCR signaling. Since the first steps of GPCR signaling take place in the membrane, membrane organization is closely linked to signaling and is therefore eventually important for drug design. A recent study could indeed show that targeting a drug to the membrane can improve its efficacy (198).

While the overall mechanism of GPCR signaling is well understood, the putative role of membrane organization remains unknown. In the canonical model for GPCR signaling, the signaling cascade is initiated by the binding of an agonist, see Fig. 6.1 a. Upon binding the receptor undergoes a conformational change (it becomes active), Fig. 6.1 b, and gains the ability to interact with its G protein, Fig. 6.1 c. The G protein is then in turn activated and the cascade processes downstream, Fig. 6.1 d. The interaction between G protein and receptor is known to happen on the same time scale as the conformational change of the receptor (145). In other words, the receptor G protein interaction happens very quickly, almost instantaneously, after ligand binding (146). That suggest that G protein and receptor are precoupled. However, experimental attempts to verify this give contradictory results (145, 147, 148). The existence of pre-coupling is therefore still actively discussed (199). Alternatively, receptor and G protein could be co-localized in small membrane domains. This would also significantly reduce the time necessary to interact. Such signaling platforms could furthermore play a role in receptor desensitization and internalization (13).

In recent years several mechanisms for the formation of membrane domains have been discussed. In addition to structures like caveolae and clathrin coated pits, lipid phase separation (lipid rafts) (9, 10, 18, 19, 157, 200) and the underlying actin cortex (picket fence model) (29, 35) were hypothesized to give rise to microscopic structure.

Since this structure influences the diffusion behavior of membrane proteins, receptor movement is an excellent readout for the involvement of

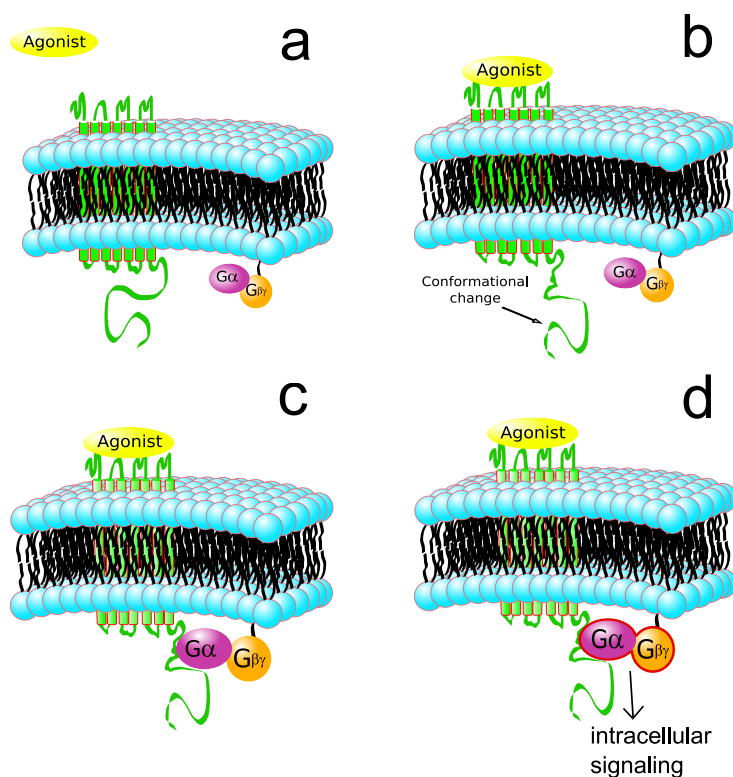


Figure 6.1
Model for GPCR signaling

membrane organization during signaling. The typical size of membrane domains requires methods that can assess molecular motion on a nanometer length scale and a millisecond time scale. These requirements make single molecule experiments the method of choice (98), optical probes used to tag the protein of interest can be localized with down to a few nanometer precision at a temporal resolution of a few milliseconds (see (111) (Chap. 5 of this thesis) and references therein).

For the measurement of receptor motion, fluorescent proteins turn out to be suitable optical tags. They can be fused to the receptor and guarantee therefore a one-to-one labeling ratio. In contrast to most other probes they can be attached to the cytosolic part of the receptor which reduces interference with ligand binding. Fluorescent proteins are also small com-

pared to other conventional probes like gold beads, labeled antibodies or quantum dots. However, the complex photophysics of these proteins (109) was long considered a serious hurdle for their use. Indeed, blinking and bleaching render the construction of single molecule trajectories difficult. In order to overcome these problems, we developed particle image correlation spectroscopy (PICS). In contrast to conventional particle tracking methods, PICS allows the robust determination of mean squared displacements (MSDs) and eventually diffusion coefficients without any a priori knowledge about diffusion speeds. In fact, the photophysics of fluorescent proteins can even be exploited in the following way. Fluorescent proteins can enter long lived dark states or a reversible bleached state which show no fluorescence. However, they recover to a fluorescent state on time scales from milliseconds to seconds (109). In conventional tracking this is considered problematic because the molecule cannot be followed while it is dark. The reconstruction of a trajectory is therefore very difficult. Since PICS does not require uninterrupted trajectories, long lived dark states or reversible bleached states actually extend the period of time over which the receptor diffusion can be accessed.

Here we present the application of PICS to the signaling of the adenosine A₁ receptor. With the help of stimulation experiments on living cells we address the role of membrane heterogeneity in the signaling process. We compare the results in living cells to the receptor movement in cell blebs to find out if the cytoskeleton is responsible for some of the observed effects. We furthermore observe the change in diffusion behavior after decoupling of receptor and G protein to answer the question whether there is receptor G-protein precoupling.

6.2 Materials and Methods

6.2.1 Single-molecule microscopy

The experimental setup for single-molecule imaging has been described in detail previously (131). Briefly, the microscope (Axiovert 100; Zeiss, Oberkochen Germany) was equipped with a 100x oil-immersion objective (NA=1.4, Zeiss, Oberkochen, Germany). The samples were illuminated for 3 ms by an Ar⁺ laser (Spectra Physics, Mountain View, CA, USA) at wavelength of 514nm. The illumination intensity was set to $2 \pm 0.2 \text{ kW/cm}^2$. Use of an appropriate filter combination (DCLP530, ET550/50m or HQ570/80, Chroma Technology, Brattleboro, USA) per-

mitted the detection of individual fluorophores by a liquid nitrogen cooled slow-scan CCD camera system (Princeton Instruments, Trenton, NY, USA). The total detection efficiency of the experimental setup was $\eta = 0.12$. For the experiments the camera was run in the kinetics-mode, permitting the recording of 8-10 images in sequence before reading out. The time between consecutive images (time lag) was set to 5-50 ms. 25-100 sequences (of 8-10 images) per cell were obtained for each time lag.

For the observation of the mobility of individual eYFP-A₁ molecules, CHO cells adhered to glass slides were mounted onto the microscope and kept in phosphate buffered saline (DPBS + CaCl₂ + MgCl₂, Gibco Invitrogen, Carlsbad, USA) at 37.5°C for the experiments on decoupling the G-protein from the receptor and 26°C for the agonist stimulation experiments (see below). In the case of the decoupling experiments pertussis toxin from *Bordetella pertussis* (PTX) was added to a final concentration of 100 ng/ml (see below). In the agonist stimulation experiments 2-chloro-N⁶-cyclo-pentyladenosine (CCPA) was added to a final concentration of 400nM (see below)

The focus of the microscope was set to the dorsal surface membrane of individual cells (depth of focus $\approx 1\mu m$). The density of fluorescent proteins on the plasma membrane of selected transfected cells was below $1\mu m^{-2}$ to permit imaging of individual fluorophores. Molecule positions were determined with an accuracy of $\approx 42 nm$.

6.2.2 Particle Image Correlation Spectroscopy (PICS)

The reconstruction of trajectories from molecule positions is severely hampered by the blinking and photobleaching of eYFP (110). Therefore we use an alternative analysis method, Particle Image Correlation Spectroscopy (PICS), described in detail elsewhere (111) (Chap. 5 of this thesis). In short, the cross-correlation between single-molecule positions at two different times is calculated. Subsequently, the linear contribution from uncorrelated molecules in close proximity is subtracted. This results in the cumulative distribution function $P(l, \Delta t)$ for the length l of diffusion steps during the time lag Δt . For each time lag P is fitted to a two fraction

model

$$P(l, \Delta t) = \alpha \left(1 - \exp \left(-\frac{l^2}{sd_1(\Delta t)} \right) \right) + (1 - \alpha) \left(1 - \exp \left(-\frac{l^2}{sd_2(\Delta t)} \right) \right) \quad (6.1)$$

where the free parameters are $sd_1(\Delta t)$ and $sd_2(\Delta t)$, the square displacements of the two fractions, and the fraction size α . We choose α to be the size of the faster fraction. A mean value of the fraction size α is determined from the results for longer time lags (50 - 250 ms) where the fit is most trustworthy. The data is then refitted with α fixed to the mean value just established. This decreases the number of free parameters to 2 ($sd_1(\Delta t)$ and $sd_2(\Delta t)$) and therefore improves the quality of the fit.

6.2.3 Analysis of MSDs (Mean square displacements)

The mean square displacements of the two fractions $sd_1(\Delta t)$ and $sd_2(\Delta t)$ are subsequently fitted to a walking diffusion model (133), see Fig. 6.3. In this model a random walker diffuses quickly with D_{micro} within a domain of size L which in turn moves slowly with D_{macro} . The model is fully equivalent to hopping diffusion where the pickets of a protein fence hinder diffusion on a macroscopic scale. The MSD is given by

$$sd(\Delta t) = \frac{L^2}{3} \left[1 - \exp \left(-\frac{12D_{\text{micro}}\Delta t}{L^2} \right) \right] + 4D_{\text{macro}}\Delta t \quad (6.2)$$

Fit parameters are the microscopic and macroscopic diffusion coefficients D_{micro} and D_{macro} and the domain size L . This model gives a very good description of the data.

6.2.4 Cell culture

For all experiments a Chinese Hamster Ovary (CHO) cell line (clone D3) stably expressing the human adenosine A₁ YFP receptor construct (201, 202) was used. Cells were cultured in DMEM:F12 1:1 medium supplemented with streptomycin (100 $\mu\text{g/ml}$), penicillin (100 U/ml) and 10% new born calf serum in a 7% CO₂ humidified atmosphere at 37°C. Cells were used for 25-30 passages and were transferred every 4 days.

For microscopy cells were cultured on cover glass slides (Assistent, Karl Hecht KG, Sondheim, Germany). For most experiments healthy looking cells were used (see Fig. 6.2, left). For comparison, some measurements were taken on cell blebs, expelled from untreated, apoptotic cells (see Fig. 6.2, right).

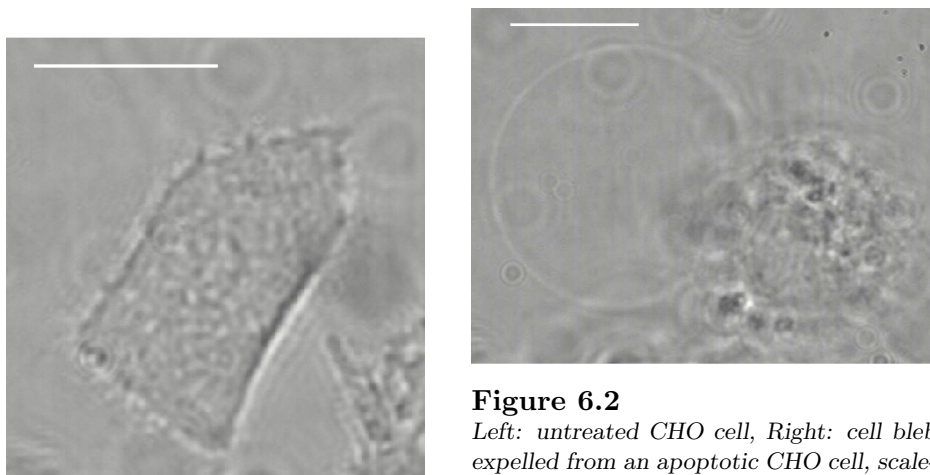


Figure 6.2

Left: untreated CHO cell, Right: cell bleb expelled from an apoptotic CHO cell, scale-bar: 10 μ m

6.2.5 Agonist stimulation assay

The activation of the adenosine A_1 receptor was achieved by the addition of 2-chloro- N^6 -cyclo-pentyladenosine (CCPA) to a final concentration of 400nM. CCPA is known to be a potent agonist of the adenosine A_1 receptor (197, 201). Since the binding affinity of CCPA is 6.4nM (197), all receptors should be activated at the CCPA concentration used. The receptor mobility was measured before stimulation and after 20 minutes of continuous stimulation.

6.2.6 Decoupling of G-protein with Pertussis toxin

Cells were incubated for 16h (basically overnight) and during observation with 100 ng/ml pertussis toxin from *Bordetella pertussis* (PTX) (Sigma-Aldrich,) Pertussis toxin is known to decouple the GPCR from its G-protein by catalysing the ADP-ribosylation of the G-protein α subunit(203).

6.3 Results

We performed single molecule microscopy experiments on the adenosine A₁ receptor in living CHO cells. For each experimental situation we determined the mean squared displacement (MSD) of the receptor with respect to time by particle image correlation spectroscopy (PICS).

Throughout all experiments the MSD is clearly non-linear, see e.g. Fig. 6.5, which reflects the heterogeneity of the membrane. On small length scales (D_{micro}) diffusion is considerably faster than on longer length scales (D_{macro}). Therefore, we use a walking diffusion model to describe the data, see Fig. 6.3. By fitting of Eq. 6.2 we determine D_{micro} and D_{macro} and the domain size L . The results for all experimental situations are summarized in Table 6.1.

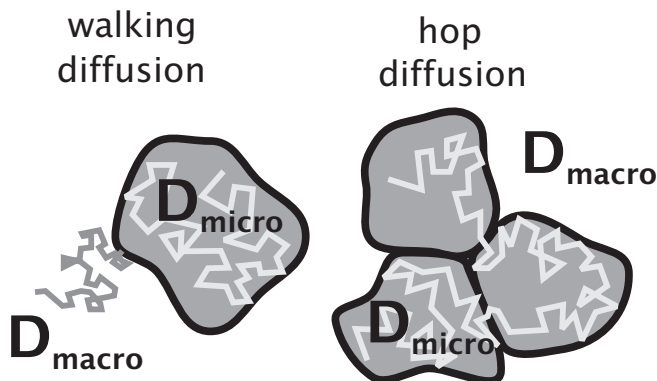


Figure 6.3

Walking diffusion (left) and hopping diffusion (right) both give rise to different diffusion coefficients on different length scales.

In addition to the non-linearity of the MSDs we find that the diffusion behavior of the receptor cannot be described by a single MSD. Throughout all experiments on living cells we find two receptor fractions with different diffusion behaviors. As can be seen from Table 6.1, microscopic and macroscopic diffusion coefficients differ at least by a factor of 3. Therefore the two fractions are dubbed "slow" and "fast" respectively. We find the size of the fast fraction to be on average $73\% \pm 2\%$. We find $D_{\text{fast}} 0.47 \pm 0.12$ and $D_{\text{slow}} 0.1 \pm 0.02$ at 37.5°C and $D_{\text{fast}} 0.15 \pm 0.28$ and $D_{\text{slow}} 0.05 \pm 0.03$ at 26°C .

6.3.1 The activated receptor translocates to microdomains

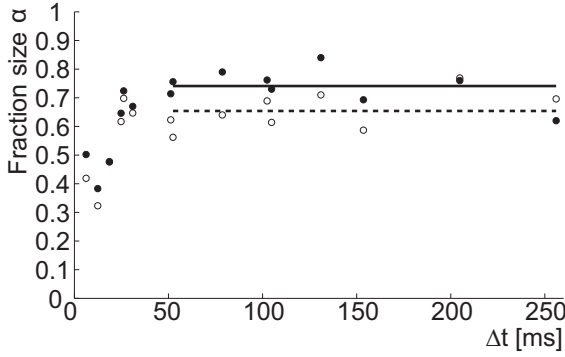


Figure 6.4

Relative size of the fast fraction (α) for the control (solid circles) and after 20 minutes of agonist stimulation (open circles). Average of the interval 50 - 256 ms gives $\alpha = 0.74 \pm 0.02$ for the control (solid line) and $\alpha = 0.65 \pm 0.02$ after stimulation (dashed line)

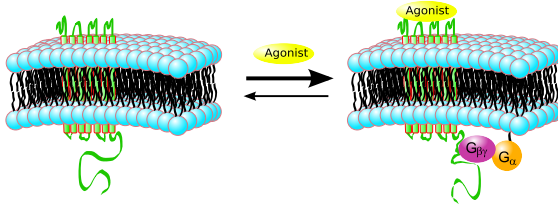
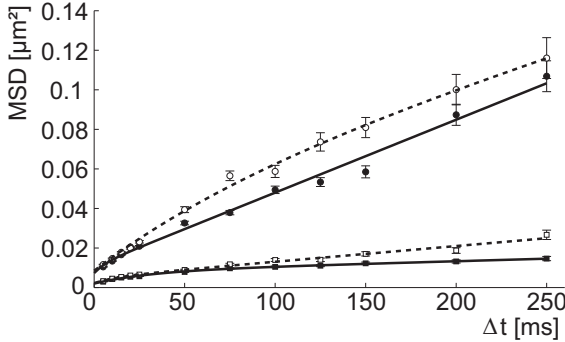


Figure 6.5

Mean square displacements of the control (solid circles: fast fraction, solid squares: slow fraction) and after 20 minutes of agonist stimulation (open circles: fast fraction, open squares: slow fraction). The lines are fits of a walking diffusion model (Eq. (6.2)) (solid lines: control, dashed lines: after stimulation). The results of the fits are given in Table 6.1).



In order to identify the two different fractions we stimulate the receptor with the agonist CCPA. Stimulation shifts the equilibrium to the activated receptor. We find that agonist stimulation decreases the size of the fast fraction α from $74\% \pm 2\%$ to $65\% \pm 2\%$, see Fig. 6.4, while the microscopic and macroscopic diffusion coefficients stay approximately the same, see Fig. 6.5 and Table 6.1. Consequently, the activated receptor must be part

of the slow fraction. The change in conformation of the receptor or the size difference between the plain receptor and the receptor-G protein complex cannot explain the difference in diffusion speed between the fast and the slow fraction (204, 205). Therefore, the receptor-G protein complex must be either localized in membrane microdomains with a higher membrane viscosity or associated with the cytoskeleton. Especially in the latter case the G-protein, which protrudes into the cytosol, might be responsible for the slow speed of the activated receptor. In our measurements we can retrieve the domain size from the walking diffusion model and we find a domain size of about 130 nm for the slow fraction and 250 nm for the fast fraction.

6.3.2 The observed microdomains are related to the cytoskeleton

To determine the connection of the membrane microdomains to the cytoskeleton we repeated the control experiment (i.e. unstimulated) in cell blebs. In cell blebs, which appear during apoptosis, the cytoskeleton is detached from the membrane. It has been observed that the lateral diffusion speed of certain membrane receptors is increased (206), which was ascribed to the release of lateral constraints. In our experiments we find very similar effects, see Fig.6.6 and Table 6.1. The diffusion coefficient is increased by a factor of 6 compared to the fast fraction of the previous experiments and there is no slow fraction anymore. This means that for the domains observed in our experiments the cytoskeleton is essential.

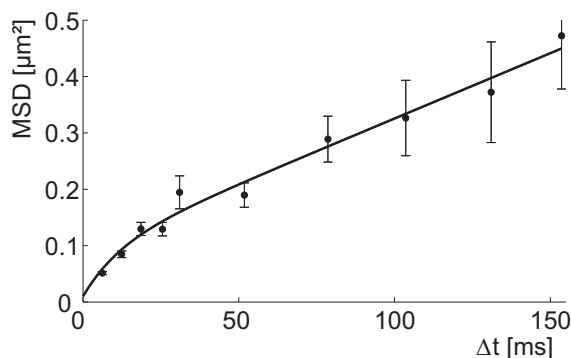


Figure 6.6

Mean square displacements of the receptor diffusion in a cell bleb (solid circles). The line is a fit of a walking diffusion model (Eq. (6.2)) The result of the fit is given in Table 6.1).

6.3.3 The receptor is partially precoupled to its G-protein

On top of the the localization in domains, G-protein and receptor could still be precoupled. Furthermore it is still unclear if coupling to the G-protein causes the slow receptor fraction to be slow. To address these questions we decouple putatively precoupled complexes by applying pertussis toxin (PTX).

Decoupling of the receptor from its G-protein causes the fast fraction α to grow, see Fig. 6.7, from $71\% \pm 2\%$ to $78\% \pm 1\%$, while the other diffusion parameters do not change, see Fig. 6.8 and Table 6.1. This result is in agreement with the stimulation experiment in which the opposite effect was observed upon receptor stimulation. Consequently, at least 7% of the receptors is precoupled to the G-protein.

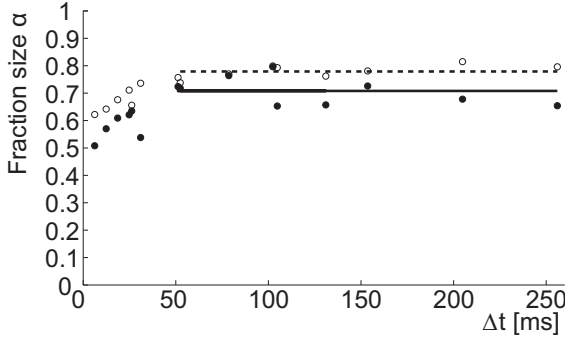
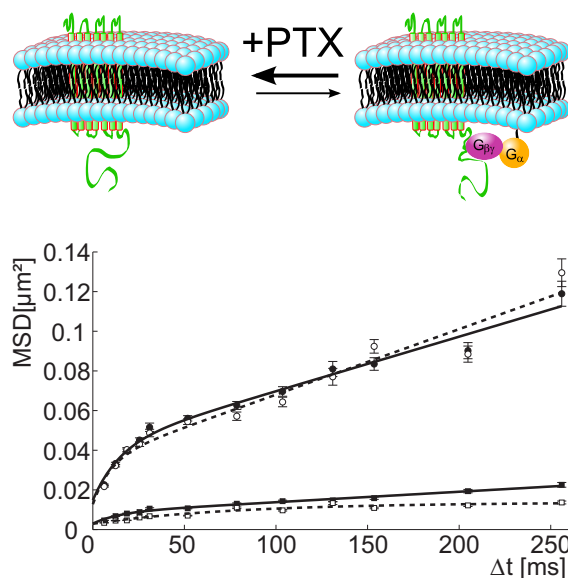


Figure 6.7

Relative size of the fast fraction (α) for the control (solid circles) and after PTX treatment (open circles). Average of the interval 50 - 256 ms gives $\alpha = 0.71 \pm 0.02$ for the control (solid line) and $\alpha = 0.78 \pm 0.01$ after PTX treatment (dashed line).

6.4 Discussion

In the present work we show that membrane heterogeneity influences adenosine A_1 receptor signaling. Consistently, we find highly non-linear MSDs and two receptor fractions which differ in diffusion coefficient. Two different diffusion coefficient were already found in earlier experiments by Briddon et al. (207) on an antagonist bound to the adenosine A_1 receptor ($D_{\text{fast}} = 0.43 \mu\text{m}^2/\text{s}$, $D_{\text{slow}} = 0.05 \mu\text{m}^2/\text{s}$). Since these results were obtained by FCS in small membrane areas, the values found are comparable to the microscopic diffusion coefficients found in our experiments, $D_{\text{fast}} 0.47 \pm 0.12$ and $D_{\text{slow}} 0.1 \pm 0.02$ at 37.5°C and $D_{\text{fast}} 0.15 \pm 0.28$ and $D_{\text{slow}} 0.05 \pm 0.03$ at 26°C . Briddon et al. ascribed the slow fraction to antagonist molecules bound nonspecifically to the membrane. They ad-

**Figure 6.8**

Mean square displacements of the control (solid circles: fast fraction, solid squares: slow fraction) and after PTX treatment (open circles: fast fraction, open squares: slow fraction). The lines are fits of walking diffusion model (Eq. (6.2)) (solid lines: control, dashed lines: after PTX treatment). The results of the fits are given in Table 6.1).

	fraction	α	D_{micro} [$\mu\text{m}^2/\text{s}$]	D_{macro} [$\mu\text{m}^2/\text{s}$]	L [nm]
control	fast	0.71 ± 0.02	0.47 ± 0.12	0.07 ± 0.01	287 ± 20
	slow		0.10 ± 0.02	0.01 ± 0.001	129 ± 7
PTX overnight	fast	0.78 ± 0.01	0.58 ± 0.35	0.08 ± 0.01	258 ± 31
	slow		0.03 ± 0.005	0.001 ± 0.006	173 ± 64
control	fast	0.74 ± 0.02	0.15 ± 0.28	0.09 ± 0.004	107 ± 35
	slow		0.05 ± 0.003	0.006 ± 0.0006	135 ± 5
20 min stimulation	fast	0.65 ± 0.02	0.10 ± 0.02	0.07 ± 0.02	337 ± 133
	slow		0.05 ± 0.03	0.02 ± 0.002	93 ± 17
Blebs			1.76 ± 0.91	0.58 ± 0.07	501 ± 86

Table 6.1

Overview of results. α size of fast fraction, D_{micro} microscopic diffusion coefficient, D_{macro} macroscopic diffusion coefficient, L domain size

mitted, however, that more rigid membrane domains could also be responsible for the slowed diffusion. Our results for the movement of the receptor show that this is indeed the case. The difference in diffusion speed between the fast and the slow fraction is too big to be explained by a change in conformation of the receptor or the size difference between the plain receptor and the receptor-G protein complex(204, 205). Consequently, membrane microdomains with a higher membrane viscosity or interaction

with the cytoskeleton must be responsible for the slowed diffusion. The conclusion is in qualitative agreement with results on the A₃ receptor in CHO cells (208). Cordeaux et al. found two different fractions where the slower fraction is identified with receptors partitioned in membrane microdomains. The size of those domains was not determined. In this work we obtain the domain sizes from the walking diffusion model and find 130 nm for the slow fraction and 250 nm for the fast fraction. These values are in agreement with scanning force microscopy experiments on living CHO cells by Lucius et al. (209). They find two populations of pits with mean diameters of around 100 nm and 200 nm. The smaller structures are speculated to be caveolae. With this interpretation our findings are consistent with earlier results by Escriche et al. (210). Their experiments show that the adenosine A₁ receptor translocates to caveolae after ligand binding. Correspondingly, we find that the slow receptor fraction increases after ligand binding by 9 percent points.

In our experiments on cell blebs, in which the actin cortex is detached from the membrane, membrane microstructure is no longer observable. This indicates that the cytoskeleton is essential for the observed heterogeneity. However, the cytoskeleton does not necessarily directly give rise to receptor confinement as assumed in the picket fence model (29). It might rather assist the assembly of heterogeneities like caveolae or clathrin coated pits and hinder the movement of those domains.

Furthermore, our experiments show that coupling of the receptor with its G protein plays an important role in the interaction with membrane heterogeneities. Upon decoupling of receptor and G protein, the fast receptor fraction increases by 7 percent points. From this, we can conclude that 1. There is precoupling between the receptor and its G protein and 2. The G protein is responsible for the interaction of the complex with membrane domains. In earlier experiments by Briddon et al. (207) faster diffusion was observed upon treatment with an antagonist. Given that an antagonist effectively causes decoupling of G-protein and receptor this result is consistent with our findings.

Comparison of our results to other GPCRs (211) show that signaling mechanisms vary, even within the same receptor class. Charalambous et al. (211) show that the mobility of the adenosine A_{2A} receptor is unaffected by agonist binding and they do not detect a significant amount of precoupling. They furthermore find that the adenosine A_{2A} receptor is localized in microdomains which are sensitive to cholesterol depletion.

These different influences of membrane organization on GPCR signaling might eventually lead to the development of highly specific drugs, which are targeted to the membrane (198).

The new insights obtained in this work were made possible by a novel technology: particle image correlation spectroscopy can be successfully used to elucidate the relevance of spatial membrane organization for signaling networks. It provides quantitative results about diffusion coefficients and confinement sizes which are vital parameters for models describing such networks.

CHAPTER 7

COUNTING AUTOFLUORESCENT PROTEINS *in vivo*

The formation of protein complexes or clusters in the plasma membrane is crucial for cell signaling and other biological processes. Therefore, it is valuable to follow complex formation in vivo. Typically, autofluorescent proteins are genetically fused to the proteins of interest to make their interaction visible. With highly sensitive single-molecule microscopy it is possible to detect single proteins and protein clusters despite autofluorescent cellular background. By measuring the fluorescence intensity of a diffraction limited spot, the number of proteins in that spot can be determined. However, the poor photophysical stability of fluorescent proteins hampers this straightforward approach. Also the presence of noise and autofluorescent background – unavoidable in live cell recordings – influence the measurement. Here we present solutions to these problems and show that the number of proteins in a diffraction limited spot can be determined in vivo in an accurate and robust way. By quantification of the number of YFP molecules in diffraction limited spots we confirm that the membrane anchor of human H-ras heterogeneously distributes in the plasma membrane. Our description of the single YFP intensity distribution therefore provides an accurate approach for quantitative in vivo investigations of protein cluster formations.

7.1 Introduction

A prominent example for the importance of protein complex formation is found in plasma membrane located signaling cascades. Here receptor molecules such as the Toll-like-receptor 3 (212) or the epidermal growth factor receptor (213) form dimers upon binding to their respective ligands. In addition to the formation of true dimers or oligomers, also protein clusters and lipid domains lead to heterogeneities in the spatial distribution of specific proteins in the plasma membrane and are known to have an effect on the dynamics of the reactions they are involved in. The small GTPase H-ras has been shown to confine to such small membrane domains upon its activation (131). Clearly, the demand for the determination of cluster formations with sufficient temporal resolution in the context of living cells is high. Quite a number of techniques have been addressed to this problem, namely bioluminescence resonance energy transfer (214), bimolecular fluorescence complementation (215), number and brightness mapping (216), image correlation spectroscopy (217), confocal laser scanning microscopy with fast scanning (218) and photon counting histograms (219). These techniques all measure ensembles of molecules, making assumptions about their collective behavior including thermal equilibrium and spatial homogeneity. The validity of such assumptions, which are potentially violated in the context of a living cell, is difficult to prove. Furthermore, many of these techniques require exact knowledge about experimental parameters, like e.g. the point spread function of the microscope, which the results might sensitively depend on.

Single-molecule experiments, on the other hand, mostly depend on universal properties of fluorescent tags and inherently possess the aspired sensitivity. It was shown *in vitro* that the number of molecules can be determined from the intensity of attached fluorophores (121). Both, the use of fluorescent proteins, which exhibit complex photophysics (109), and the presence of high noise make this type of analysis more challenging in the *in vivo* context. In a recent study Ulbrich et al. demonstrated that molecule numbers can be assessed *in vivo* from the bleaching steps of autofluorescent proteins (106). This method requires the selection of intensity trajectories that show the expected step-wise decrease of fluorescence. Another approach, introduced by Cognet et al. (107), is to collect all emitted photons until photobleaching, such that fluorescence intermittency is averaged out. This method essentially uses photon counting histograms (141) to determine the underlying distribution of molecule numbers. Here we report

on the adaptation of this single-molecule method, which was demonstrated *in vitro* (107, 121), to autofluorescent proteins in living cells. We put the approach taken intuitively by Cognet et al. on firm theoretical grounds using semi-classical Mandel theory (220). Our theoretical result allows us to choose experimental parameters for a faithful measurement of single-molecule intensity distributions. Furthermore, we address the problems arising from an autofluorescent background, which are especially severe in living cells. In particular, the probability to detect a fluorophore in a noisy background depends on the intensity of the fluorophore and therefore modulates measured intensity distributions. We quantify this detection probability and its influence on intensity measurements. We verified our theoretical predictions by measuring the integrated intensities of single YFP molecules in living cells under experimental conditions where the molecules bleach within the illumination time. We show that the resulting distributions can be described by a simple one-parameter model, which allows for the quantification of molecule numbers by established methods (121).

Finally, we apply our method to the membrane distribution of the H-Ras membrane anchor. While measurements at low spatial densities of the protein yield a strictly monomeric distribution, only slight increases in H-Ras density cause evident increases of the dimeric fraction. Assuming a random spatial distribution of H-Ras, such a density dependent effect would be expected only at much higher concentrations. We therefore have to assume a non-random distribution of H-ras. Hence, we are able to confirm the results of an earlier study (150) that this membrane anchor clusters on length scales below the width of the point spread function ($\approx 200nm$), exclusively by using information from measurements of single molecule intensities.

Taken together, our method to accurately and quantitatively describe the intensity distribution of YFP-fusion proteins *in vivo* is suited, to characterize the spatial distribution of membrane proteins based on membrane heterogeneities or domain formations on the length scale of or below the diffraction limit. While inherently the method is not able to report the exact size of such domains, it makes up for this lack of spatial resolution by providing temporal resolution. With our method, the status of domain presence can in principle be monitored many times in the course of a biological reaction, such as a signaling event. In the same way, the presence or formation of true protein complexes (i.e. dimers, trimers or

higher multimers) would lead to intensity distributions, from which our method could extract the stoichiometry and its changes. However, as our results show, membrane heterogeneity has to be taken into account when true complex formation is to be measured.

7.2 Materials and Methods

7.2.1 DNA constructs

The protocol for the preparation of the DNA constructs was previously described in detail in (150). The DNA sequence encoding the 10 C-terminal amino acids of human H-Ras (GCMSCCKVLS), which includes the CAAX motif, was inserted in frame at the C-terminus of the enhanced yellow-fluorescent protein (EYFP, S65G/S72A/T203Y) coding sequence using two complementary synthetic oligonucleotides (Isogen Bioscience, Maarsse, The Netherlands). The integrity of the reading frame of the resulting EYFP-C10HRas construct was verified by sequence analysis. For expression in mammalian cells, the complete coding sequence of EYFP-C10HRas was cloned into the pcDNA3.1 vector (Invitrogen, Groningen, The Netherlands).

7.2.2 Cell culture

For all experiments a Chinese Hamster Ovary (CHO) cell line (clone D3) was used. Cells were cultured in DMEM:F12 1:1 medium supplemented with streptomycin ($100\mu\text{g}/\text{ml}$), penicillin ($100\text{U}/\text{ml}$) and 10% new born calf serum in a 7% CO_2 humidified atmosphere at 37°C . Cells were used for 25–30 passages and were transferred every 4 days. For microscopy cells were cultured on cover glass slides (Assistent, Karl Hecht KG, Sondheim Germany) and transfected with 250ng DNA and $3\mu\text{l}$ FUGENE HD (Roche Molecular Biochemicals, Indianapolis, USA) per glass slide (1h incubation time). For a convenient expression level cells were used 3–4 days after transfection.

7.2.3 Single-molecule microscopy

The experimental setup for single-molecule imaging has been described in detail previously (150). Briefly, the microscope (Axiovert 100; Zeiss, Oberkochen Germany) was equipped with a 100x oil-immersion objective

(NA=1.4, Zeiss, Oberkochen, Germany). The samples were illuminated for $T = 50ms$ by an Ar^+ laser (Spectra Physics, Mountain View, CA, USA) at a wavelength of $514nm$. The illumination intensity was set to $3 \pm 0.3kW/cm^2$. A circular diaphragm was introduced in the back focal plane of the tube lens to confine the illumination area. This results in a flat laser illumination profile. An appropriate filter combination (DCLP530, ET550/50m, Chroma Technology, Brattleboro, USA) permitted the detection of individual fluorophores by a liquid nitrogen cooled slow-scan CCD camera system (Princeton Instruments, Trenton, NY, USA). The total detection efficiency of the imaging optics was $\eta_o = 0.12$. The time between consecutive images (time lag, Δt) was set to $254ms$. Typically, 4000-8000 images were obtained per cell. For the observation of the intensity of individual EYFP-CAAX molecules, CHO cells adhered to glass slides were mounted onto the microscope and kept in phosphate buffered saline (PBS: 150mM NaCl, 10mM Na_2HPO_4/NaH_2PO_4 , pH 7.4) at $37^\circ C$. The focus of the microscope was set to the ventral surface membrane of individual cells (depth of focus $\approx 1\mu m$). The density of fluorescent proteins on the plasma membrane of selected transfected cells was less than $1\mu m^{-2}$ to permit imaging of individual fluorophores. According to (110) the bleaching time τ_{bl} for the used laser intensity $I_{ill} = 3kW/cm^2$ is $10.4ms$. The probability that a single EYFP bleaches within the illumination/integration time $T = 50ms$ is therefore $p_{bl} > 99\%$ (110). In other words, a single EYFP is bleached within the illumination time. The bleaching rate $k_{bl} = 1/\tau_{bl}$ is well separated from $1/T$ ($k_{bl} \approx 0.2T^{-1}$) and therefore the simplified model described below (see Eq. 7.1) is applicable. The expected photon emission rate expected from results in (110) is $F = 775$ photons/ms. Therefore, $N = \eta_o \tau_{bl} F = 967$ photons are expected to be detected during the average lifetime τ_{bl} of the fluorophore, where the detection efficiency is $\eta_o = 0.12$.

7.2.4 Image analysis

At first, the autofluorescent background is subtracted from the raw images. The background subtracted images are subsequently filtered with a Gaussian whose width corresponds to the width of the point spread function (PSF) of the microscope. This procedure optimizes the signal to noise ratio. The positions of the pixels whose value after filtering exceeds a certain multiple of the noise are used as initial values for the fitting of a 2D Gaussian in the unfiltered image. From this fit, position, width and

integrated intensity of the single molecule signal are determined. More details can be found in the supplements (Sec. 7.B).

7.3 Results and Discussion

If several fluorescent molecules are colocalized on a length scale of $\lesssim 200nm$, their fluorescence signal will be a single diffraction limited spot in a widefield microscope. In the following we will refer to a certain number of molecules in a diffraction limited spot as monomer, dimer, trimer, ... irrespective of the origin of colocalization: the molecules might e.g. be part of a stable complex or transiently reside in the same nanoscopic domain. Although the molecules cannot be resolved, it is possible to infer their number from the integrated fluorescence signal. Since the number of molecules cannot be calculated from a single signal (due to noise), it is necessary to analyze distributions of fluorescence intensities of many diffraction limited spots. To first approximation, the total fluorescence signal integrated over the diffraction limited spot should be linearly proportional to the number of molecules. In experiments this simple relationship does not hold due to the complex photophysics of fluorescent tags and the data analysis process, as detailed in the subsequent sections.

7.3.1 Blinking and bleaching of fluorescent proteins

YFP and other autofluorescent proteins are popular tags for biomolecules *in vivo* because of their ease of use and the guaranteed 1:1 labeling ratio. Unfortunately, fluorescent proteins exhibit complex photophysics: they are known to blink, i.e. switch transiently between fluorescent and non-fluorescent states, and bleach fairly quickly. This poor photostability can make it difficult to infer molecule numbers from the fluorescence signal. We illustrate the photophysics of a fluorescent protein with a 3-state model derived in Sec. 7.C.1, see inset to Fig. 7.1A. In this model the fluorophore switches between 'on' and 'off' with a rate k and bleaches with a rate k_{bl} from the 'on' states. Only in the 'on' state the protein emits photons with a mean rate $\bar{I}$. It cannot return to a fluorescent state once it is bleached. Fig. 7.1 shows the number of photons emitted by a single fluorophore during illumination time T calculated from the 3-state model. In Fig. 7.1A the influence of blinking is illustrated. Since the photon emission rate in the 'on' state is set to $\bar{I} = 100/T$ the mean of the distribution is

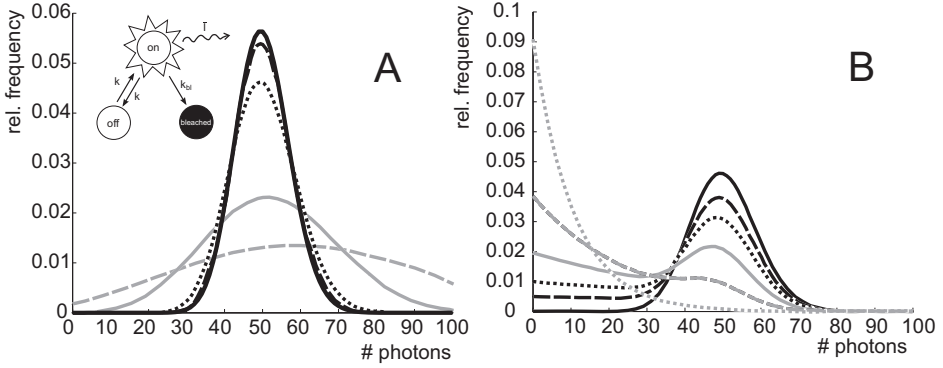


Figure 7.1

Photophysical model for blinking behavior of YFP. Inset panel A: Schematic representation of the model. The fluorophore switches between 'on' and 'off' with a rate k and bleaches with a rate k_{bl} from the 'on' states. Only in the 'on' state the protein emits photons with a mean rate $\bar{I}$. Once bleached it cannot return to a fluorescent state. A) Influence of blinking for negligible bleaching. Relative frequency of numbers of photons emitted by a single fluorophore during illumination time T . The bleaching rate k_{bl} is in all cases $k_{bl} = (10000T)^{-1}$, the rate of photon emission in the 'on' state is $f = 100/T$. The blinking rate k is $k \rightarrow \infty$ (solid black line, limit given by Poisson distribution), $k = (0.3333T)^{-1}$ (dashed black line), $k = (0.1T)^{-1}$ (dotted black line), $k = (0.01T)^{-1}$ (solid grey line), $k = (0.002T)^{-1}$ (dashed grey line). B) Influence of bleaching for fixed blinking rate. Relative frequency of numbers of photons emitted by a single fluorophore during illumination time T . The blinking rate k is in all cases $k = (0.01T)^{-1}$, the rate of photon emission in the 'on' state is $f = 100/T$. The bleaching rate k_{bl} is $k_{bl} = (10^4T)^{-1}$ (solid black line), $k_{bl} = (2T)^{-1}$ (dashed black line), $k_{bl} = T^{-1}$ (dotted black line), $k_{bl} = (0.5T)^{-1}$ (solid grey line), $k_{bl} = (0.25T)^{-1}$ (dashed grey line), $k_{bl} = (0.1T)^{-1}$ (dotted grey line).

approximately 50, at least for high blinking rates. Clearly, the width of the distribution increases with decreasing blinking rate. Note that even for infinitely fast blinking the distribution has a finite width. This minimal width is due to the fact that photon emission is a stochastic process. The variance of the Poisson distribution, which describes this process, is equal to the mean (here: 50), so the minimal width (standard deviation) is $\sqrt{50}$. For very slow blinking, the mean shifts to the right (dashed grey line in Fig. 7.1), if the fluorophore is initially 'on'. Both effects make it difficult to distinguish the monomer with mean intensity 50 from the dimer, which would have a mean intensity of 100. As with blinking, bleaching strongly distorts the intensity distributions, (Fig. 7.1B). While for small bleaching rates the distribution shows a clear local maximum (black solid line in

Fig. 7.1B), the distribution follows an exponential decay for fast bleaching (black dotted grey line in Fig. 7.1B).

7.3.2 Robust intensity distributions

For both blinking and bleaching the shape of the distributions changes the most with varying k and k_{bl} when the time scales for blinking ($1/k$) and bleaching ($1/k_{\text{bl}}$) are comparable to the illumination time T .

Since both k and k_{bl} sensitively depend on many experimental parameters (illumination intensity, local pH, ...) the observed variability in the intensity distributions prevents a robust assessment of molecule numbers. Along the lines of ideas developed by Cognet et al. (107), we therefore propose to use long illumination times T such that $T \gg 1/k \gg 1/k_{\text{bl}}$.

In that case the intensity distribution assumes a very simple form, which is independent of the value of k . In Sec. 7.C.1 we show that for large T the distribution of the number n of photons detected during time T is

$$p(n; N) = \frac{1}{N} \left(1 + \frac{1}{N}\right)^{-(n+1)} \quad (7.1)$$

where N is the mean number of photons detected. $N = \eta_d \eta_o \bar{I} k_{\text{bl}}^{-1}$ where η_d and η_o are the quantum yield of the detector and the detection efficiency of the imaging optics, respectively. This intensity distribution is not influenced by blinking and depends on k_{bl} in a defined way, making it a good starting point for the measurement of molecule intensities. Below we show that the intensities of single YFP molecules follow this distribution if T is sufficiently large.

The distribution of the intensity of a dimer (i.e. two fluorophores in a diffraction limited spot) $p_2(n; N)$ is obtained from the convolution of Eq. 7.1 with itself (121):

$$p_2(n; N) = \sum_{n'=0}^{\infty} p(n - n') p(n') = \frac{n+1}{(1+N)^2} \left(1 + \frac{1}{N}\right)^{-n} \quad (7.2)$$

Continued convolution with $p(n; N)$ (Eq. 7.1) gives the distribution for higher multimers, like e.g. a trimer

$$p_3(n; N) = \sum_{n'=0}^{\infty} p(n - n') p_2(n') = \frac{(n+1)((n/2)+1)}{(1+N)^3} \left(1 + \frac{1}{N}\right)^{-n} \quad (7.3)$$

In Fig. 7.2 the intensity distributions for a monomer, dimer and trimer with the same mean number of detected photons N (per fluorophore) are compared.

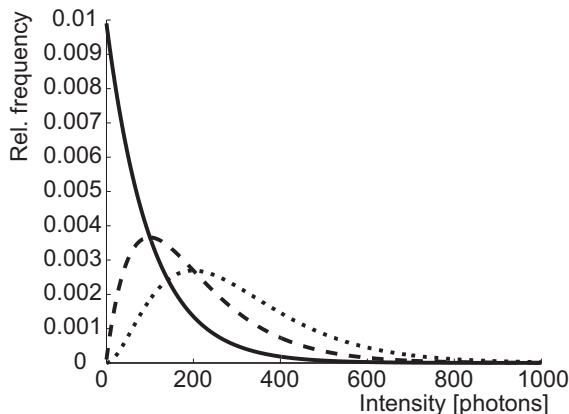


Figure 7.2

Intensity distribution $p(n; N)$ for the monomer (solid line) given by Eq. 7.1 and for the dimer (dashed line) and trimer (dotted line) obtained from convolution of $p(n; N)$ with itself. The mean number of detected photons is $N = 100$ in all cases.

In principle one could use the distributions derived so far to fit a measured intensity distribution and determine the fraction of monomers, dimers, etc. However, experimental factors modulate the measured intensity distributions as detailed in the subsequent section.

7.3.3 Detection probability

In the preceding section we showed that the intensity distribution of a single fluorophore follows an exponential decay for long illumination times T (Eq. 7.1), which means that a significant fraction of the molecules has a very small intensity. However, such dim molecules cannot be detected due to the presence of noise. This experimental noise, which originates from photon counting statistics, the detection apparatus and background autofluorescence of the cell, is unavoidable. To increase the signal to noise ratio (SNR) the acquired image is filtered with a Gaussian of width w , which should equal the width of the present signals, as prescribed by optimal filtering theory (see Sec. 7.B). Still, even after filtering, a threshold has to be defined to distinguish noise from a real single-molecule signal: only those pixels that exceed the noise by a threshold factor t (i.e. $\text{SNR} > t$) are considered to be part of potential single-molecule signals. If the threshold factor t is chosen too large, few single molecules will be detected, if it is too small, however, noise will be falsely identified as

single-molecule signals.

To quantify the influence of thresholding on intensity distributions we derived in Sec. 7.B the probability to detect a single molecule signal of width w and integrated intensity A at a noise level σ and threshold t

$$p_{\text{det}}^{\text{max}}(A; \sigma, w, t) = \frac{1}{2} \left(1 + \text{erf} \left(\frac{A}{\sqrt{8\pi\sigma w}} - \frac{t}{\sqrt{2}} \right) \right) \quad (7.4)$$

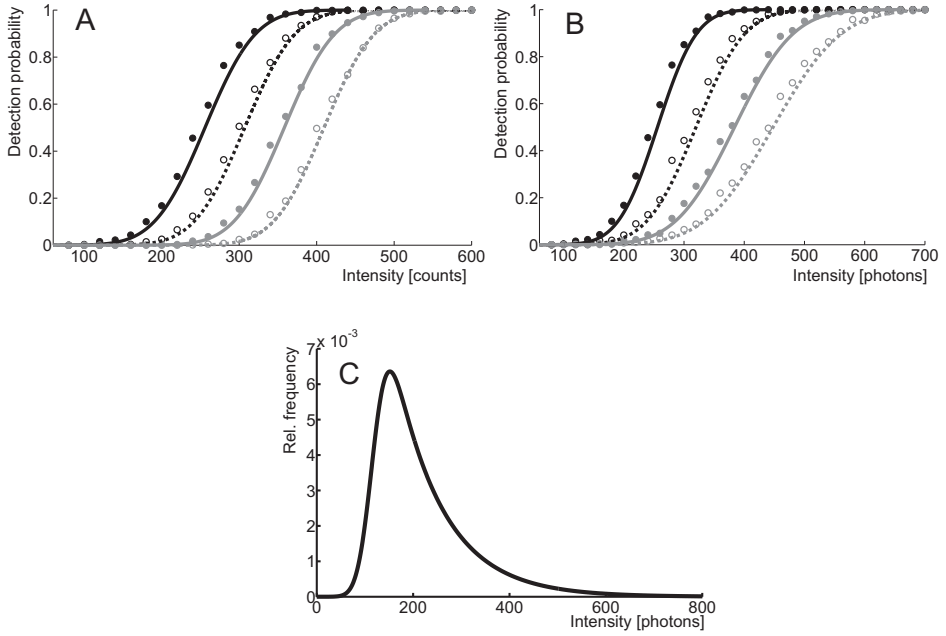
where t is the threshold imposed on the SNR after filtering the image with a Gaussian filter of width w . Fig. 7.3A and Fig. 7.3B show very good agreement between this theoretical result and simulated data. The slight systematic underestimation of the simulated detection probability is probably due to the fact that only the maximum (i.e. brightest pixel) of a single-molecule signal is considered in the model (Sec. 7.B). This pixel has the highest chance to be detected (i.e. to exceed the threshold) in the presence of noise. Other, adjacent pixels, which belong to the same single-molecule signal and by definition are less bright, also slightly contribute to the detection probability of the whole signal. Their contribution has been neglected in the derived model.

With the help of Eq. 7.4 we can now theoretically determine the shape of *measured* intensity distributions. Measured intensity distributions are products of the distribution of the emitted intensities (Eq. 7.1) and the detection probability (Eq. 7.4). An example for such a distribution is given in Fig. 7.3C. Since the detection probability goes to 0 for small intensities, measured intensity distributions always have a peak at finite intensities, despite the fact that the underlying distribution of emitted intensities is *maximal* for small intensities. In Sec. 7.3.4 we will show that experimentally determined intensity distributions indeed have the predicted shape shown in Fig. 7.3C.

To find the optimal value for the threshold t we have to balance the number of rejected single-molecule signals, which increases with t , with the number of false positives (i.e. noise accepted as signal), which decreases with t . To predict the number of false positives we calculated the probability $p_{\text{false}}(t)$ to falsely detect a single molecule in a pixel with only noise at threshold t (see also Sec. 7.B).

$$p_{\text{false}}(t) = \frac{1}{2} \left(1 + \text{erf} \left(-\frac{t}{\sqrt{2}} \right) \right) \quad (7.5)$$

In an image with M pixels roughly $M \cdot p_{\text{false}}(t)$ noise peaks are falsely detected as single molecules signals, if the pixels can be considered in-

**Figure 7.3**

A) Detection probability determined from simulations for a threshold t of 5 (black solid circles), 6 (black open circles), 7 (grey solid circles) and 8 (grey open circles) respectively at noise level $\sigma = 20$ and signal width $w = 0.7pxl$. The lines give the detection probability predicted by Eq. 7.4 for a threshold t of 5 (black solid line), 6 (black dashed line), 7 (grey solid line) and 8 (grey dashed line). B) Detection probability determined from simulations for noise levels σ of 20 (black solid circles), 25 (black open circles), 30 (grey solid circles) and 35 (grey open circles) respectively at a threshold t of 5 and signal width $w = 0.7pxl$. The lines give the detection probability predicted by Eq. 7.4 for a noise level σ of 20 (black solid line), 25 (black dashed line), 30 (grey solid line) and 35 (grey dashed line). C) Complete intensity distribution for a single fluorophore (monomer) given by Eq. 7.46. This distribution is calculated as the product of the monomer distribution (Eq. 7.1) and the detection probability (Eq. 7.4) and subsequent normalization to 1. The assumed parameters are: number of detected photons $N = 100$, noise level $\sigma = 10$, threshold $t = 5$, signal width $w = 0.7pxl$.

dependent. We define ϵ as the maximal allowed ratio of false positives ($M \cdot p_{\text{false}}(t)$) to all detected signals N_{signals} : $M p_{\text{false}}(t) < \epsilon N_{\text{signals}}$. This definition leads to an upper limit for t : $t > \sqrt{2} \operatorname{erf}^{-1}(1 - 2\epsilon(N_{\text{signals}}/M))$. For example with $\epsilon = 0.01$, $M = 50^2$ and typically $N_{\text{signals}} = 10$ we get $t \gtrsim 4$. Since the total number of detected signals depends on N_{signals} and will decrease with t , t should not be chosen too large to avoid loss of data.

7.3.4 Experimental validation

To verify our theoretical derivations we performed single-molecule fluorescence experiments on EYFP molecules in living CHO cells. EYFP was tagged to the membrane anchor of H-Ras, which resulted in a membrane localization of the EYFP and thereby greatly facilitated the measurements. We measured the intensities of ≈ 23000 single-molecule signals for an illumination time of $T = 50ms$. We estimated from earlier experiments (see Sec. 7.2) that an illumination time of $50ms$ is several times bigger than the bleaching time expected at the illumination intensities used. To ensure that there was only one EYFP in each diffraction limited spot the signal density was kept very low ($\rho < 0.2\mu m^{-2}$). Fig. 7.4A shows the obtained intensity distributions. As expected from theory (Eq. 7.1) the distribution approximately follows an exponential decay. The measured mean number of photons of $N = 837 \pm 3$ is roughly in agreement with the value expected from earlier results on EYFP (see Sec. 7.2), which was 967. The single molecule signals were obtained with a threshold factor of $t = 3$ which minimizes the influence of the detection probability on the intensity distribution. However, only signal intensities above 1000 *photons* were used, which roughly corresponds to a threshold of $t = 15$. Hence, the number of false positives due to noise is minimized. To show that thresholding influences intensity distributions in the way we derived above, we present in Fig. 7.4B several intensity distributions based on the same raw data, which differ by the threshold factor t . In principle, these distributions should be the product of the distribution of emitted photons (Eq. 7.1), with the mean number of photons N determined above, see Fig. 7.4A), and the detection probability at a certain threshold factor t and noise level σ . This product is given in Eq. 7.46. The noise level σ is not constant in our experiments but varies between images and, more strongly, between different cells. Therefore the detection probability has to be determined as the average over all acquired images using the noise levels in those images

$$\bar{p}_{\text{det}}^{\text{max}}(A; w, t) = \frac{1}{N_{\text{images}}} \sum_i^{N_{\text{images}}} p_{\text{det}}^{\text{max}}(A; \sigma_i, w, t) \quad (7.6)$$

where σ_i is the noise level in image i and N_{images} is the number of acquired images.

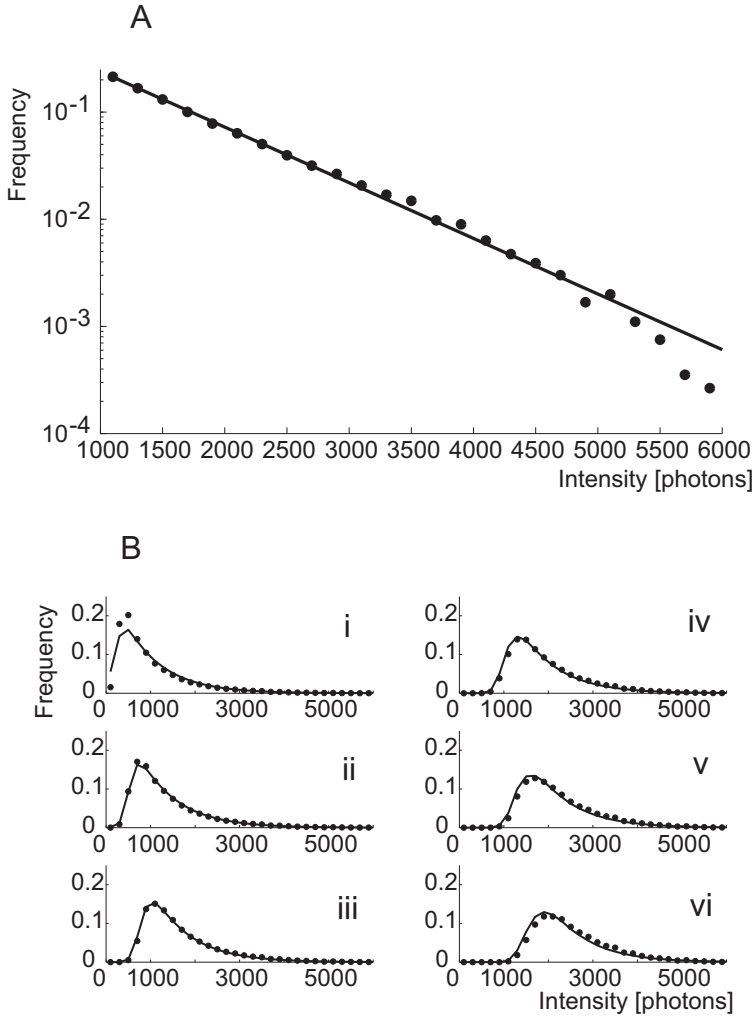


Figure 7.4

Intensity distribution of single EYFPs on the membrane of living CHO cells. A) Experimental intensity distribution of the intensities of 22615 single-molecule fluorescence signals (solid circles). A one-parameter fit of Eq. 7.1 (solid line) to the experimental intensity distribution for single EYFPs gives $N = 837 \pm 3$ photons. The width w of the single molecule signals was restricted to the interval $0.64 - 0.81 \text{ pixel}$, the width of the Gaussian filter was $r = 0.72 \text{ pxl}$, $\text{pxl} = 220 \text{ nm}$. The threshold factor was $t = 3$ and only signals with an intensity bigger than 1000 photons were used. B) Influence of thresholding on the shape of the intensity distributions. The raw data is the same as in panel A, but the threshold factor t is varied. $t = 5, 10, 15, 20, 25, 30$ from i) to vi) respectively. The experimental data (solid circles) is compared to the full theoretical distribution Eq. 7.46 (solid line). The mean number of detected photons N was determined above from a restricted data set (see panel A), the detection probability was calculated by integration over the noise levels found in the raw images.)

The noise levels were estimated as described in Sec. 7.B. The resulting detection probability $\bar{p}_{\text{det}}^{\text{max}}(A; w, t)$ is then multiplied with the distribution of emitted intensities (Eq. 7.1) to get the full theoretical description of the measured intensity distributions (solid lines in Fig. 7.4B). The measured distributions nicely follow the theoretical expressions determined in this way.

7.3.5 Clustering due to membrane heterogeneity

The measurements presented in the previous section were performed at low signal densities ($< 0.2\mu\text{m}^{-2}$) to ensure that there was only one single YFP molecule per diffraction limited spot. In subsequent measurements at increased signal densities we observed that the intensity distributions are shifted to higher intensities, see Fig. 7.5. This shift is due to the presence of several molecules in one diffraction limited spot (multimers). To quantify the amounts of monomers, dimers and higher multimers we compare the intensity distributions at various densities of single molecule signals to the intensity distribution of the monomer(121). For simplicity we assume here that only monomers and dimers are present and describe the measured distributions as a weighted sum of the intensity distribution of a monomer $p(n; N)$ and a dimer $p_2(n; N)$

$$p_{\text{total}}(n) = \alpha p(n; N) + (1 - \alpha)p_2(n; N). \quad (7.7)$$

$p(n; N)$ and $p_2(n; N)$ were presented in Sec. 7.3.2 (see Sec. 7.C.1 for the derivation). N , the average number of detected photons, is determined from the monomer distribution as described in the previous section which leaves the fraction of monomers α as a free fit parameter. Fig. 7.5 shows an example for a fit of this model to experimental data. We find that α decreases quickly with increasing signal density, see inset of Fig. 7.5. Strikingly, there are many more dimers than expected for a uniform distribution of molecules at such low densities ($< 1\mu\text{m}^{-2}$, see the model derived in Sec. 7.C.1). In fact, this is in agreement with earlier results on the used construct (150) where a certain fraction of the molecules were shown to exhibit confined diffusion in $\approx 200\text{nm}$ domains. Consequently, one would expect colocalization of molecules even at low densities. This result has important implications. On the one hand intensity can be used as a readout for membrane heterogeneity. On the other hand, this result shows that membrane heterogeneity has to be taken into account when

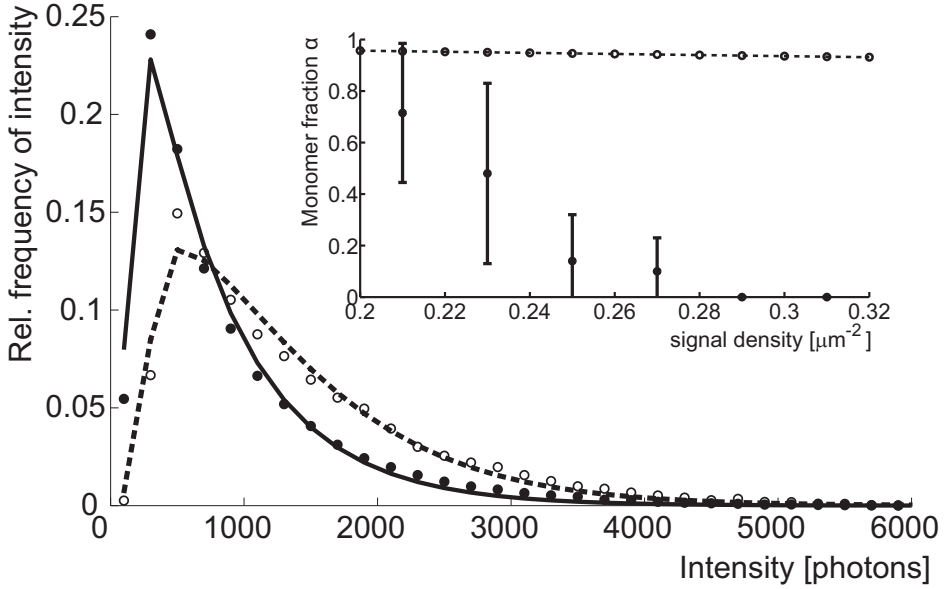


Figure 7.5

Influence of membrane heterogeneity on the intensity distribution of EYFP in the membrane of living CHO cells. The solid circles give the intensity distribution at low signal densities ($< 0.2 \mu\text{m}^{-2}$), already shown in Fig. 7.4. The solid line is the corresponding theoretically expected distribution obtained as detailed above. This distribution is compared to one taken at a signal density of $0.25 \mu\text{m}^{-2}$ (open circles). The threshold factor is $t = 3$ for both distributions. The visible shift to higher intensities with increased signal densities is due to the presence of multimers (i.e. several molecules colocalized in a diffraction limited spot). A fit to Eq. 7.7 (dashed line) gives that at this density the fraction of monomers is $\alpha = 0.14$. Inset: Fraction of monomers versus density of single molecule signals (solid circles). The error was determined as standard deviation calculated from all data sets used in a certain bin. The open circles show the theoretically expected monomer fraction for a uniform distribution of molecules, see Eq. 7.49.

true complex formation (in contrast to mere colocalization) is to be measured.

7.3.6 Limitations and errors

The method presented here requires long illumination times T . Mobile molecules in living cells can be observed only for a limited time, which sets an upper boundary for T . In our experiments this did not present a severe limitation but it might be e.g. for molecules which diffuse more

quickly.

Secondly, the diffusion of molecules during illumination broadens the observed single-molecule signals and might render fitting the signals difficult, especially if signal density is high. To quantify the influence of diffusion we estimate the expected signal width and the broadening caused by diffusion. For a single YFP with emission maximum at $\lambda = 527nm$ and a numerical aperture of the microscope objective of $NA = 1.4$ we would expect the full width half maximum of a signal to be $\approx 2 \cdot \lambda / (2NA) = 376nm$ which corresponds to a signal width of $w_{PSF} = 160nm$. The signal width is defined as the width of a gaussian which is fit the point spread function (PSF) of the microscope, see Sec. 7.B. Additionally, the movement of the molecule in the period before bleaching leads to a broadening of the peak. With a bleaching time of $\tau_{bl} = 10.4ms$ (see Sec. 7.2) and a diffusion coefficient of roughly $D = 0.8\mu m^2/s$ (150), the total signal width is, according to (221), $w = \sqrt{(w_{PSF})^2 + D\tau_{bl}} = 178nm$. This broadening effect is small and does not hamper the applicability of the method in our experiments. In general, the signal width puts an upper limit on the density of signals: two molecules which come closer to each other than w_{PSF} cannot be resolved.

In addition to long illumination times T our method also requires that the bleaching time scale is much longer than time scale of any blinking (see Sec. 7.C.1). In our experiments blinking is obviously fast enough because we observe the exponential decay predicted for well separated time scales. Different fluorophores might have blinking rates which are comparable to the bleaching rate. Such molecules cannot be used with our method.

We estimated the error for the measurement of the monomer fraction in Sec. 7.3.5 with the help of simulations. In particular, we assume that the mean number of emitted photons N is known and randomly generate $N_{signals}$ signals with intensities drawn from the distribution Eq. 7.7. The randomly drawn intensities are binned in equally sized bins and the resulting distribution is normalized. This distribution is fit with Eq. 7.7 with α as the only free fit parameter. The whole procedure is repeated 100 times for each set of parameters and the error is determined as the standard deviation $\Delta\alpha$ over the 100 values obtained for α . Fig. 7.6 compares the influence of the experimental parameters on the relative error of α determined by these simulations. Fig. 7.6A shows that $\Delta\alpha$ approximately scales like $\propto 1/\sqrt{N_{signals}}$, which means that the accuracy can always be increased by measuring more signals. As expected the relative

error of α increases with decreasing α . In Fig. 7.6B we show that $\Delta\alpha$ scales approximately like $\propto 1/\alpha$. If the bin size is sufficiently small, $\Delta\alpha$ is independent of the mean number of emitted photons N , see Fig. 7.6C. If the bin size is too large, all signals will fall in one (or a few bin), which makes fitting of the distribution impossible. Conversely, $\Delta\alpha$ is independent of the bin size, see Fig. 7.6C, unless the mean number of emitted photons N is small. In that case the bin size has to be chosen sufficiently small. The relative error we expect for the parameters of the experiment

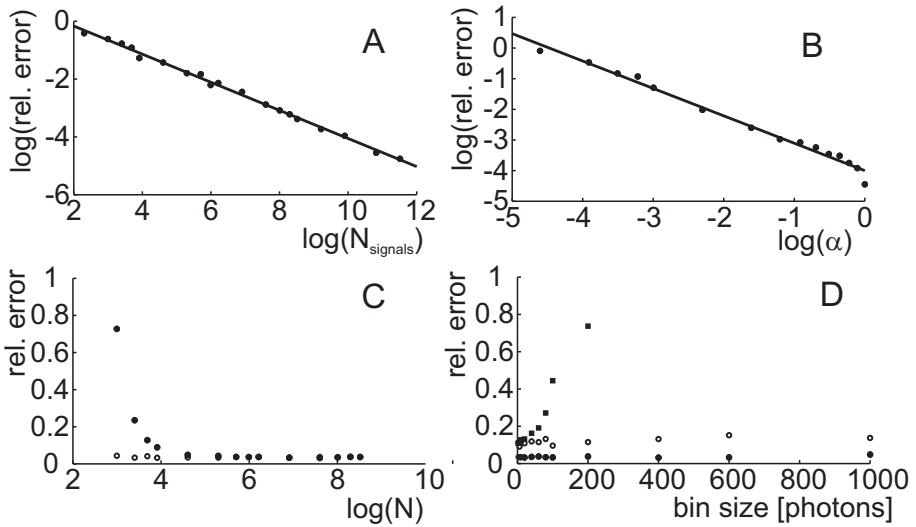


Figure 7.6

Accuracy of the measurement of the monomer fraction α determined from simulations. A) Dependence of the relative error of α (solid circles) on the number of detected signals N_{signals} . A linear fit to the data in the log-log graph (solid line) has a slope of -0.49. The bin size is 200, $N = 500$, $\alpha = 0.5$. B) Dependence of the relative error of α (solid circles) on α . A linear fit to the data in the log-log graph (solid line) has a slope of -0.89. The bin size is 200, $N = 500$, $N_{\text{signals}} = 5000$. C) Dependence of the relative error of α on the mean number of emitted photons N . Solid circles correspond to simulations with a bin size of 200, the open circles to simulations with a bin size of 20. $N_{\text{signals}} = 5000$, $\alpha = 0.5$ D) Dependence of the relative error of α on the bin size b . The solid circles correspond to simulations with $N_{\text{signals}} = 5000$ and $N = 500$, the open circles to simulations with $N_{\text{signals}} = 500$ and $N = 500$ and the solid squares to simulations with $N_{\text{signals}} = 500$ and $N = 20$

presented above ($N = 837$, bin size 200, $N_{\text{signals}} \approx 20000$) ranges from

0.08 for $\alpha = 0.1$ to 0.01 for $\alpha = 1$. The errors we observe in experiments are much larger, see inset of Fig. 7.5. The reason for that is twofold: Our simulations do not account for biological variability which is probably considerable. Secondly, we combined data sets with different signal densities in bins with a bin size of $0.02\mu m^{-2}$, see inset of Fig. 7.5. Since the monomer fraction is decreasing quickly with signal density, heterogeneity within a bin contributes significantly to the reported error.

7.4 Conclusion

We have shown that, despite the many challenges presented by living cells, single-molecule intensities can be measured in a robust way. We have put such measurements on firm mathematical and analytical grounds. In particular, we have analyzed and minimized the influence of noise and the noise related thresholding procedure. We have shown experimentally that using long illumination times overcomes the problems arising from single-molecule blinking and bleaching. The resulting intensity distributions are well described by a simple, quantitative model. Finally, we have quantified the amount of clustering of a certain membrane protein due to membrane heterogeneity. We hope that our work paves the way for accurate *in vivo* measurements of protein complex and cluster formation. In particular, since our method is based on imaging, it should allow for the construction of cell-wide stoichiometry maps.

7.5 Acknowledgement

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7.A Image processing and analysis

7.A.1 Definitions

The image taken by the CCD camera $s(x, y)$ is composed of the following contributions

$$s(x, y) = \sum_i G_i(x, y) + B(x, y) + n_0(x, y) \quad (7.8)$$

where G_i are the single-molecule signals at positions (x_i, y_i) , B is the autofluorescent background and, $n_0(x, y)$ is a constant Gaussian noise (e.g. read-out noise).

Since the signal is obtained by counting single-photons, the probability for a certain signal $G_i(x, y)$ coming from a single molecule is given by a Poisson distribution with mean $g_i(x, y)$

$$p(G_i(x, y)) = \frac{g_i(x, y)^{G_i(x, y)}}{G_i(x, y)!} \cdot \exp[-g_i(x, y)] \quad (7.9)$$

where $g_i(x, y)$ is the point spread function (PSF) of the microscope centered at molecule position (x_i, y_i) . The PSF, which is ideally given by a 2D Airy-function, is most commonly approximated by a 2D Gaussian

$$g_i(x, y) = \frac{A}{2\pi w^2} \cdot \exp\left[-\frac{(x - x_i)^2 + (y - y_i)^2}{2w^2}\right] \quad (7.10)$$

Correspondingly the contribution of the background is given by

$$p(B(x, y)) = \frac{b(x, y)^{B(x, y)}}{B(x, y)!} \cdot \exp[-b(x, y)] \quad (7.11)$$

where $b(x, y)$ is given by the product of the laser profile and the autofluorescence coming from the cell.

The constant noise contribution $n_0(x, y)$ is assumed to be a normal distribution with mean 0 and standard deviation σ_0 .

7.A.2 Autofluorescent background subtraction

We assume that $b(x, y)$ only fluctuates weakly on the length scale of the signal width w at the time scale of the time lag between frames Δt . Therefore, a low pass Fourier filtering of single images or a sliding minimum of an

image stack gives approximately $b(x, y)$ (see below). After subtraction of $b(x, y)$ the background is approximately flat. We further assume that the influence of the background is so weak that the remaining shot-noise can be described by a normal distribution with standard deviation $\sqrt{b(x, y)}$. These simplifications lead to the following estimation of the signal

$$s(x, y) = \sum_i G_i(x, y) + n(x, y) \quad (7.12)$$

where $n(x, y)$ is distributed normally with mean 0 and standard deviation $\sigma(x, y) = \sqrt{\sigma_0^2 + b(x, y)}$. Since we assumed that $b(x, y)$ is approximately constant on the length scale w , we can also write $\sigma = \sqrt{\sigma_0^2 + \bar{b}}$ where $\bar{b}$ is the background averaged over the whole image.

i) Sliding minimum filter Since experimental parameters are chosen such that single molecule signals only appear in a single frame (single molecules are bleached during integration time T) and the autofluorescent background bleaches slowly, a sliding minimum filter can be applied. In the time trace of each pixel the value of a pixel at a certain time t is exchanged for the local minimum (in time). The local minimum is defined as the minimal pixel value in a time window of width τ around t . By using a sliding minimum instead of a sliding mean, the intensity of single-molecule signals remains unchanged. The remaining noise, however, is biased towards positive values.

ii) High pass FFT filter Since the width of single molecule signals is smaller than typical length scales of autofluorescent structures, the background can be diminished by high pass Fourier filtering. As described in the following, the filter does not change positions or intensities of single molecule signals, if applied correctly. It is less effective than the sliding minimum filter but causes no bias in the remaining noise.

For the FFT filter we can estimate the influence of the filtering on the signal intensities. As already mentioned, the PSF of the microscope is approximated by a Gaussian

$$g_i(x, y) = \frac{A}{2\pi w^2} \cdot \exp \left[-\frac{(x - \hat{x}_i)^2 + (y - \hat{y}_i)^2}{2w^2} \right] \quad (7.13)$$

The Fourier transform results in a modified gaussian

$$g_i(k_x, k_y) = e^{-\frac{(2\pi w)^2(k_x^2 + k_y^2)}{2}} e^{-i2\pi(\hat{x}_i k_x + \hat{y}_i k_y)} \quad (7.14)$$

Note that the information about the width of the signal is reflected in the amplitude, and the information about the position is reflected in the phase. By changing the amplitude only, the positions are preserved. Therefore

$$h(k_x, k_y) = e^{-\frac{(2\pi v)^2(k_x^2 + k_y^2)}{2}} \Rightarrow \tilde{g}(k) = g_i(k_x, k_y) \cdot h(k_x, k_y) \quad (7.15)$$

Inverse Fourier transform yields

$$\hat{g}_i(x, y) = \frac{A}{2\pi(v^2 + w^2)} \cdot \exp\left[\frac{(x - \hat{x}_i)^2 + (y - \hat{y}_i)^2}{2(v^2 + w^2)}\right] \quad (7.16)$$

The filtered image is subsequently subtracted from the original image. $f(x, y) = g_i(x, y) - \hat{g}_i(x, y)$ If $v \gg w$ then the form of the signal is hardly distorted. Since the width of the PSF depends approximately quadratically on the z-position ($w(z) = w_0(1 + cz^2)$), filtering is more effective for out of focus light. For $z \neq 0$ the PSF and $\hat{g}_i(x, y)$ (the filtered PSF) differ less (since $w(z) > w(0)$), which results in a reduction of out of focus signal.

As an estimate for the change in intensity due to filtering the relative signal height after filtering is calculated

$$\frac{\text{height of filtered signal}}{\text{original signal height}} = \frac{\frac{1}{w^2} - \frac{1}{w^2 + v^2}}{\frac{1}{w^2}} = 1 - \left(1 + \left(\frac{v}{w}\right)^2\right)^{-1} \quad (7.17)$$

If v is too small, signal height and intensity decrease too much. For $v = 5w$ the height is still 96% of the original signal height, so $v > 5w$ is a reasonable limit. If v is too big, however, the filtering effect will be small.

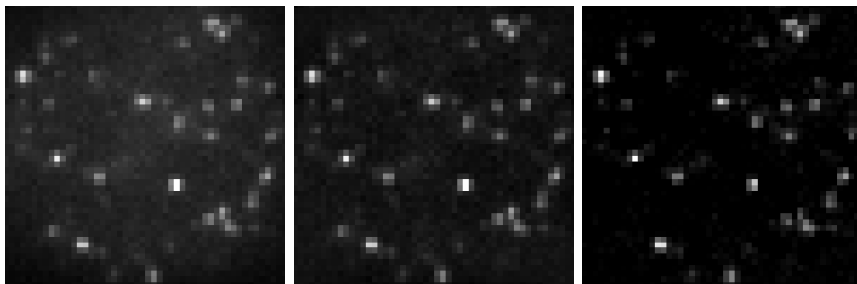
The different filters are compared in Fig. 7.7.

It is evident from Fig. 7.7 that the sliding minimum filter increases the signal to background ratio most. However, background estimation methods should be compared as well in terms of noise, offset and signal width.

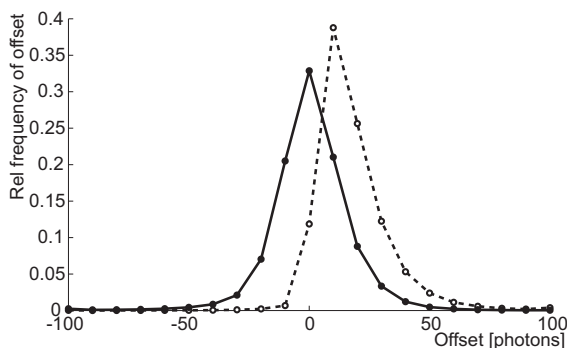
Fig. 7.8 shows that the FFT filter results in an offset that is symmetrically distributed around 0, the sliding minimum filter, however, causes a systematic shift in offset.

Also in terms of noise the sliding minimum filter is superior, which is shown in Fig. 7.9. Images filtered with the FFT method show a systematically higher noise level.

Furthermore, a difference in width of the found signals is evident in Fig. 7.10. The maximum at 1.7pixel corresponds to the signal from single

**Figure 7.7**

From left to right: raw image, image filtered with high pass FFT filter ($v = 5\text{pxl}$), image stack filtered with sliding minimum filter (window size: 2 frames)

**Figure 7.8**

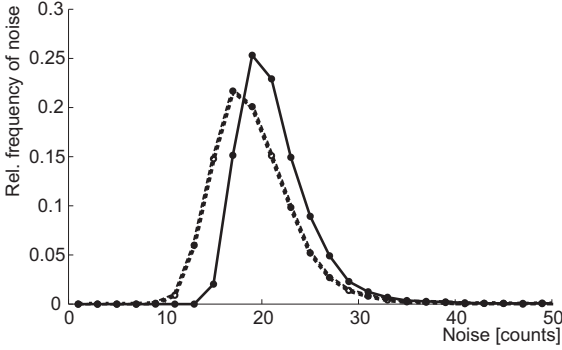
Offset distribution. Solid line: FFT filter, Dashed line: sliding minimum, threshold $t = 5$

molecules, see Sec. 7.3.6 the additional maximum around 1pxl is due to noise that is falsely identified as a single molecule (see 7.B). The FFT filter clearly results in less false positive detections.

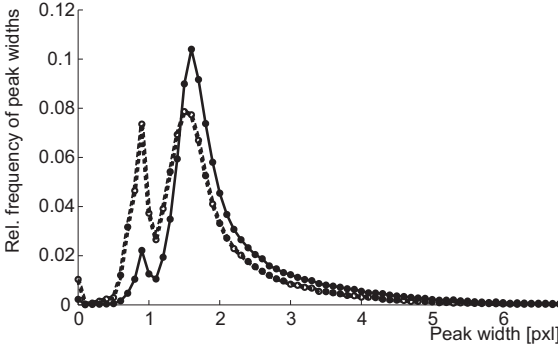
Although the sliding minimum filter gives a better signal to background ratio and less noise we chose to employ in the following only the FFT filter. It outperforms the other method in terms of offset and false positive detections.

7.B Detection probability

After background subtraction the image is filtered with an appropriate filter to optimize the signal to noise ratio (SNR). According to optimal filtering theory (142), the filter should be identical to the signal. Therefore

**Figure 7.9**

Noise distribution. Solid line: FFT filter, Dashed line: sliding minimum, threshold $t = 5$

**Figure 7.10**

Signal width distribution. Solid line: FFT filter, Dashed line: sliding minimum, threshold $t = 5$

we filter with a Gaussian of width r . The resulting SNR is

$$\text{SNR} = \frac{Ar}{\sigma\sqrt{\pi}(r^2 + w^2)} \quad (7.18)$$

where $\tilde{A} = A/(2\pi(r^2 + w^2))$ is the maximum of the signal (= signal height) after filtering and $\tilde{\sigma} = (\sigma/2\sqrt{\pi}r)$ is the noise level after filtering. The noise distribution is still normal (with mean 0 and standard deviation $\tilde{\sigma}$). For the optimal choice $r = w$

$$\text{SNR}_{\max} = \frac{A}{\sigma\sqrt{\pi}w} \quad (7.19)$$

. Notice that σ has the units [counts/pixel] so that the SNR is dimensionless. In order to distinguish between noise and signal, a threshold t is introduced. Only those pixels in the filtered image whose brightness

$I(x, y)$ exceeds the (filtered) noise level by a factor t are treated as potential molecule positions.

$$I(x, y) > t \cdot \frac{\sigma}{2\sqrt{\pi}r} \equiv t\tilde{\sigma} \quad (7.20)$$

Fig. 7.11 illustrates the thresholding procedure. Subimages of the unfil-

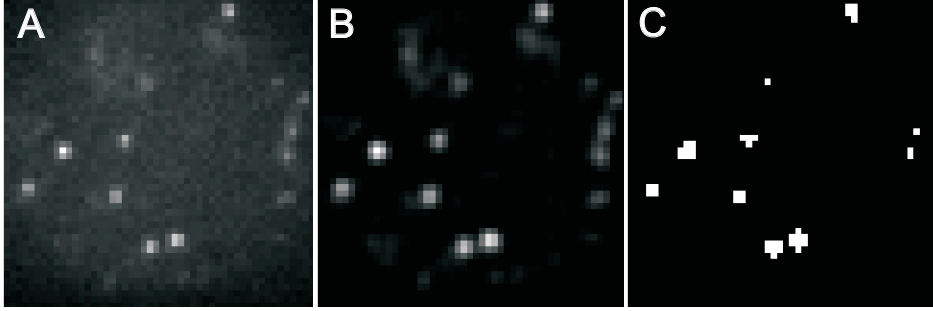


Figure 7.11

Thresholding procedure A) Raw image of single EYFP-CAAX molecules on the membrane of a living CHO cell. The noise is approximately $\sigma = 26$. The linear grey scale ranges from 0 counts (black) to 1002 counts (white). B) Image after background subtraction and filtering (i.e. correlation) with gaussian of width 0.7pxl , $\text{pxl} = 220\text{nm}$. The linear grey scale ranges from 0 counts (black) to 1473 counts (white). C) Binary image after thresholding. White pixels correspond to pixels whose value exceeds the threshold at threshold factor $t = 10$.

tered image around the pixels which were identified as potential molecule positions are then fit with the sum of a Gaussian and a constant offset $g(x, y) + \text{off}$.

We define the detection probability $p_{\text{det}}(\sigma, A)$ as the probability that for a given noise level σ a pixel with brightness $\tilde{A}$ exceeds the threshold described, so

$$p_{\text{det}}(\sigma, A) = \int_{t\tilde{\sigma}}^{\infty} da \frac{1}{2\pi\tilde{\sigma}} \exp \left[-\frac{(a - (\tilde{A} + \text{off}))^2}{2\tilde{\sigma}^2} \right] \quad (7.21)$$

Integration gives

$$p_{\text{det}}(\sigma, A) = \frac{1}{2} \left(1 + \text{erf} \left(\frac{1}{\sqrt{2}} \left(\frac{\tilde{A} + \text{off}}{\tilde{\sigma}} - t \right) \right) \right) \quad (7.22)$$

$$= \frac{1}{2} \left(1 + \text{erf} \left(\frac{1}{\sqrt{2}} \left(\frac{A + \text{off}'}{\sigma} \cdot \frac{r}{\sqrt{\pi}(r^2 + w^2)} - t \right) \right) \right) \quad (7.23)$$

with the error function

$$\operatorname{erf}(x) = \frac{2}{\sqrt{\pi}} \int_0^x dy \exp(-y^2) \quad (7.24)$$

and $\operatorname{off}' = \operatorname{off} \cdot 2\pi(r^2 + w^2)$. For a given σ , A and w the detection probability is maximal for the choice $r = w$, i.e. when the SNR is maximal. If we assume $\operatorname{off} \ll A$

$$p_{\text{det}}^{\text{max}}(\sigma, A) = \frac{1}{2} \left(1 + \operatorname{erf} \left(\frac{A}{\sqrt{8\pi}\sigma w} - \frac{t}{\sqrt{2}} \right) \right) \quad (7.25)$$

Obviously the integrated signal intensity A should be as big as possible and noise level σ and signal width w should be as small as possible to maximize the detection probability. The threshold parameter t should be as small as possible to detect as many molecules as possible. However, with decreasing t the probability $p_{\text{false}}(\sigma)$ that a noise peak is falsely detected as a signal is growing. In complete analogy to the above derivation the probability $p_{\text{false}}(\sigma)$ is

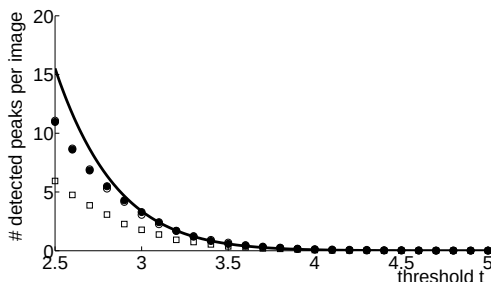
$$p_{\text{false}}(t) = \frac{1}{2} \left(1 + \operatorname{erf} \left(-\frac{t}{\sqrt{2}} \right) \right) \quad (7.26)$$

In an image with M pixels roughly $M \cdot p_{\text{false}}(t)$ noise peaks are falsely detected as signals. As shown in Fig. 7.12 this is a good estimation for a filter width of $r = 0.7\text{pxl}$. For wider Gaussian filters the theoretical formula overestimate the number of false positives. In this case, the pixels are not independent any more, so there are fewer false positive detections than expected. Also for small thresholds t , theory and simulation differ. Here the number of false detections becomes so high, that they overlap and cannot be distinguished anymore. In any case the theoretical expression Eq. 7.26 is an upper bound for the number of false positives. It can therefore be used to safely estimate the threshold t required for a certain maximal number of false positives.

7.C Intensity distribution

7.C.1 Mandel theory

According to the semi-classical theory by Mandel (220) which is used also in Photon Counting Histograms (PCH) (141) the probability to find n

**Figure 7.12**

Number of signals detected in an image with only noise, image size 50x50 pixels. Filled circles: noise level $\sigma = 50$, filter width $r = 1.7\text{pxl}$; open circles: noise level $\sigma = 20$, filter width $r = 0.7\text{pxl}$; open squares: noise level $\sigma = 20$, filter width $r = 1.1\text{pxl}$, solid line: number of false positives expected from Eq. 7.26

photons at time t with an integration/illumination time T and detector quantum yield η_d is:

$$p(n, t, T) = \int_0^\infty \frac{(\eta_d W(t))^n e^{-\eta_d W(t)}}{n!} p_{\text{inc}}(W(t), T) dW(t) \quad (7.27)$$

$$W(t) = \eta_o \int_t^{T+t} \int_A I(\mathbf{r}, t) d\mathbf{A} dt \quad (7.28)$$

$W(t)$ is the number of incident photons falling on the detector given a photon emission rate $I(\mathbf{r}, t)$, a detection efficiency of the imaging optics η_o and a detector area A . $p_{\text{inc}}(W(t), T)$ is the probability distribution of the number of incident photons $W(t)$ at time t given an illumination time T .

Here integration is performed not over the whole detector (which would be the whole CCD chip) but over the area on the chip that is covered by a single-molecule signal so that

$$W(t) = \eta_o \int_t^{T+t} I_{\text{sm}}(t) dt \quad (7.29)$$

where $I_{\text{sm}}(t)$ is the photon emission rate coming from a single molecule. T is assumed to be so long that $W(t)$ is independent of the time of measurement t : $W(t) \equiv W$. Note that this is the opposite of the limit used in PCH (141) where very short integration/illumination times T are used so that the intensity fluctuations govern the counting statistics. For long enough illumination times T the probability distribution $p_{\text{inc}}(W, T)$ can be obtained from the probability $p_{\text{fluor}}(t_{\text{on}}, T)$ that a molecule is "on", i.e. emitting photons, for a period t_{on} during the integration time T . If $\bar{I}$ is

the average number of photons emitted during periods when the molecule is "on", $p_{\text{inc}}(W, T)$ is

$$p_{\text{inc}}(W, T) = \frac{\eta_o}{\bar{I}} p_{\text{fluor}}\left(t_{\text{on}} = \frac{W}{\bar{I}}, T\right) \quad (7.30)$$

The distribution of the "on" times, $p_{\text{fluor}}(t_{\text{on}}, T)$, depends on the photophysics of the molecule. In the following two paragraphs a model for $p_{\text{fluor}}(t_{\text{on}}, T)$ that includes blinking and bleaching is derived.

7.C.2 2-state model

The 2-state model with a fluorescent "on" and a non-fluorescent "off" state was discussed in (222). In this model the molecule can switch reversibly between the "on" state and the "off" state but it never bleaches. The corresponding probability distribution for the times t_{on} in the "on" state $p_{\text{fluor}}(t_{\text{on}}, T)$ is the sum of contributions from 4 different kinds of fluorescence traces. 1. A molecule that starts in the "on" state can stay "on" during the whole illumination time T ($t_{\text{on}} = T$). The probability for such a trace is $p_{\text{on}}(0) \exp(-k_{\text{off}}T)$ where $p_{\text{on}}(0)$ is the probability that the molecule is initially "on" and k_{off} is the rate for switching from "on" to "off". 2. A molecule that starts in the "off" state can stay "off" during the whole illumination time T ($t_{\text{on}} = 0$). The probability for such a trace is $p_{\text{off}}(0) \exp(-k_{\text{on}}T)$ where $p_{\text{off}}(0)$ is the probability that the molecule is initially "off" and k_{on} is the rate for switching from "off" to "on". Since a molecule can either be "on" or "off" initially, $p_{\text{on}}(0) + p_{\text{off}}(0) = 1$ must hold. 3. A molecule that starts in the "on" state can switch between "on" and "off". The probability density for those fluorescence traces is

$$p_{\text{odd}}(t_{\text{on}}, T) = k_{\text{off}} \exp(-k_{\text{off}}t_{\text{on}} - k_{\text{on}}t_{\text{off}}) \times I_0\left(2\sqrt{k_{\text{off}}k_{\text{on}}t_{\text{on}}t_{\text{off}}}\right) \quad (7.31)$$

for an odd number of switches and

$$p_{\text{even}}(t_{\text{on}}, T) = \sqrt{k_{\text{off}}k_{\text{on}} \frac{t_{\text{on}}}{t_{\text{off}}}} \exp(-k_{\text{off}}t_{\text{on}} - k_{\text{on}}t_{\text{off}}) \times I_1\left(2\sqrt{k_{\text{off}}k_{\text{on}}t_{\text{on}}t_{\text{off}}}\right) \quad (7.32)$$

for an even number of switches. $t_{\text{off}} = T - t_{\text{on}}$ and I_0 and I_1 are modified Bessel-functions of the first kind of order 0 and 1 respectively, see (222).

The total probability for traces which start in the "on" state and switch between "on" and "off" is $p_{\text{on}}(0) (p_{\text{odd}}(t_{\text{on}}, T) + p_{\text{even}}(t_{\text{on}}, T))$. 4. The probability density for a molecule which starts in the "off" state and switches between "off" and "on" are analogous: k_{on} is interchanged with k_{off} and t_{on} is interchanged with t_{off} . The total probability density for the "on" times t_{on} is the sum of all 4 contributions

$$\begin{aligned}
 p(t_{\text{on}}, T) = & p_{\text{on}}(0) \\
 & \times \exp(-k_{\text{off}}T) \delta(T - t_{\text{on}}) + p_{\text{off}}(0) \exp(-k_{\text{on}}T) \delta(t_{\text{on}}) + \Theta(t_{\text{on}}) \Theta(T - t_{\text{on}}) \\
 & \times \exp(-k_{\text{off}}t_{\text{on}} - k_{\text{on}}t_{\text{off}}) \left[(p_{\text{on}}(0)k_{\text{off}} + p_{\text{off}}(0)k_{\text{on}}) I_0 \left(2\sqrt{k_{\text{off}}k_{\text{on}}t_{\text{on}}t_{\text{off}}} \right) \right. \\
 & \left. + (p_{\text{on}}(0)\sqrt{t_{\text{on}}/t_{\text{off}}} + p_{\text{off}}(0)\sqrt{t_{\text{off}}/t_{\text{on}}}) \sqrt{k_{\text{off}}k_{\text{on}}} I_1 \left(2\sqrt{k_{\text{off}}k_{\text{on}}t_{\text{on}}t_{\text{off}}} \right) \right]
 \end{aligned}$$

7.C.3 3-state model

We generalize the above model presented in the previous section to a 3-state model in which the fluorophore can bleach from the "on" state to a bleached state with rate k_{bl} , see Inset to Fig. 7.1A. In this model the probability distribution $p_{\text{fluor}}(t_{\text{on}}, T)$ is again given by the sum of 4 contributions. 1. A molecule starts in the "on" state and stays "on" during the whole illumination time T ($t_{\text{on}} = T$). The probability for such traces is now $p_{\text{on}}(0) \exp(-(k_{\text{off}} + k_{\text{bl}})T)$. 2. The probability that the molecule starts in the "off" state and stays "off" during T ($t_{\text{on}} = 0$) is as above: $p_{\text{off}}(0) \exp(-k_{\text{on}}T)$. 3. The contribution of traces that are switching and end in the "on" or "off" state without bleaching during the illumination time T is the same as contribution 3 of the 2-state model except for an additional factor $\exp(-k_{\text{bl}}t_{\text{on}})$. 4. A molecule can start in the "on" or "off" state and after several switching events the fluorescence trace is ended by a final bleaching event from the "on" state. The probability that a molecule is initially "on" and stays "on" until it bleaches at time t_{on} is $p_{\text{on}}(0)k_{\text{bl}} \exp(-(k_{\text{bl}} + k_{\text{off}})t_{\text{on}})$. If the molecule switches, it has to switch an even number of times if it is initially "on" and an odd number of times if it is initially "off" to end the fluorescence trace in the "on" state before bleaching. The corresponding probabilities are $p_{\text{on}}(0)p_{\text{even}}(t_{\text{on}}, t')$ and $p_{\text{off}}(0)p_{\text{odd}}(t_{\text{on}}, t')$ where t' is the point in time when the bleaching event takes place. t' lies between t_{on} (then the molecule is continuously "on" until it bleaches) and T (then t_{on} is spread over the illumination time T), see Fig. 7.13.

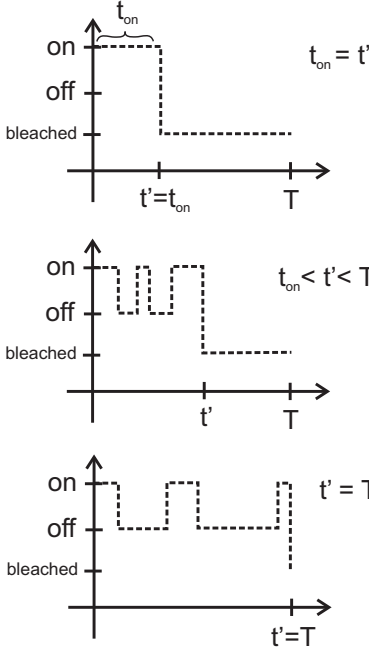
**Figure 7.13**

Illustration of fluorescence traces contributing to the probability distribution $p_{\text{fluor}}(t_{\text{on}}, T)$. In all three cases the "on" time is identical while the time point of bleaching t' is varied. In the top most trace the time of bleaching t' is identical to t_{on} , the molecule has to be continuously in the "on" state until bleaching. In the middle trace the "on" time is spread over the time until bleaching due to intermittent periods in the "off" state. In the bottom trace the molecule bleaches exactly at the end of the illumination period. The "on" time is spread over the whole illumination period T .

To properly account for all possible traces one has to integrate t' over all allowed values $t_{\text{on}} < t' < T$. Finally, the probability for a bleaching event after an "on" time of t_{on} is $k_{\text{bl}} \exp(-k_{\text{bl}} t_{\text{on}})$. In summary, the contribution of switching traces which are ended by a final bleaching event is:

$$\begin{aligned}
 & p_{\text{on}}(0) k_{\text{bl}} \exp(-(k_{\text{bl}} + k_{\text{off}}) t_{\text{on}}) \\
 & + k_{\text{bl}} \exp(-k_{\text{bl}} t_{\text{on}}) \int_{t_{\text{on}}}^T dt' (p_{\text{on}}(0) p_{\text{even}}(t_{\text{on}}, t') + p_{\text{off}}(0) p_{\text{odd}}(t_{\text{on}}, t'))
 \end{aligned} \tag{7.33}$$

Adding the contributions from the 4 different kinds of fluorescence traces

results in

$$\begin{aligned}
p(t_{\text{on}}, T) = & p_{\text{on}}(0) \exp(-(k_{\text{off}} + k_{\text{bl}})T) \delta(T - t_{\text{on}}) \\
& + p_{\text{off}}(0) \exp(-k_{\text{on}}T) \delta(t_{\text{on}}) + \Theta(t_{\text{on}}) \Theta(T - t_{\text{on}}) \left[k_{\text{bl}} \exp(-k_{\text{bl}}t_{\text{on}}) \right. \\
& \times \left(p_{\text{on}}(0) \exp(-k_{\text{off}}t_{\text{on}}) + \int_{t_{\text{on}}}^T dt' (p_{\text{on}}(0) p_{\text{even}}(t_{\text{on}}; t') + p_{\text{off}}(0) p_{\text{odd}}(t_{\text{on}}; t')) \right) \\
& + \exp(-k_{\text{bl}}t_{\text{on}}) \exp(-k_{\text{off}}t_{\text{on}} - k_{\text{on}}t_{\text{off}}) \\
& \times \left[(p_{\text{on}}(0)k_{\text{off}} + p_{\text{off}}(0)k_{\text{on}}) I_0 \left(2\sqrt{k_{\text{off}}k_{\text{on}}t_{\text{on}}t_{\text{off}}} \right) \right. \\
& \left. \left. + (p_{\text{on}}(0)\sqrt{t_{\text{on}}/t_{\text{off}}} + p_{\text{off}}(0)\sqrt{t_{\text{off}}/t_{\text{on}}}) \sqrt{k_{\text{off}}k_{\text{on}}} I_1 \left(2\sqrt{k_{\text{off}}k_{\text{on}}t_{\text{on}}t_{\text{off}}} \right) \right] \right]
\end{aligned}$$

We simplify this model by assuming that "on" and "off" rates are equal ($k_{\text{on}} = k_{\text{off}} = k$) and that the molecule is initially always in the "on" state ($p_{\text{on}}(0) = 1, p_{\text{off}}(0) = 0$).

If we use the probability distribution $p_{\text{fluor}}(t_{\text{on}}, T)$ for the 3-state model (Eq. 7.34) to calculate the distribution of incident photons $p_{\text{inc}}(W, T)$ (Eq. 7.30) and insert $p_{\text{inc}}(W, T)$ in Mandel's theory (Eq. 7.27) we obtain the probability distribution $p(n, T)$ for the number of photons detected during illumination time T . This distribution was used to illustrate the influence of blinking and bleaching in Sec. 7.3.1.

7.C.4 Robust intensity distributions

If the illumination time T is so big that bleaching is fast on the time scale set by T ($k_{\text{bl}} > 1/T$), the molecule bleaches within the illumination time T . If, additionally, blinking is faster than bleaching $k > k_{\text{bl}}$, the molecule switches frequently between the "on" and "off" state during $1/k_{\text{bl}}$ and k_{bl} is the only relevant rate. Under these conditions we can significantly simplify Eq. 7.34 to

$$p_{\text{fluor}}(t_{\text{on}}) = k_{\text{bl}} \exp(-k_{\text{bl}}t_{\text{on}}) \quad (7.34)$$

$$\Rightarrow p_{\text{inc}}(W) = \frac{k_{\text{bl}}}{\eta_o \bar{I}} \exp\left(-\frac{k_{\text{bl}}W}{\eta_o \bar{I}}\right) \quad (7.35)$$

If we insert this distribution into Mandel's formula Eq. 7.27 we get:

$$p(n, T) \equiv p(n) = \int_0^\infty \frac{(\eta_d W)^n e^{-\eta_d W}}{n!} \cdot \frac{k_{\text{bl}}}{\eta_o \bar{I}} \exp\left(-\frac{k_{\text{bl}} W}{\eta_o \bar{I}}\right) dW \quad (7.36)$$

$$= \frac{k_{\text{bl}}}{\eta_o \bar{I}} \frac{\eta_d^n}{n!} \int_0^\infty W^n e^{-\eta W} dW \quad \text{with} \quad \eta = \eta_d + \frac{k_{\text{bl}}}{\eta_o \bar{I}} \quad (7.37)$$

$$= \frac{k_{\text{bl}}}{\eta_o \bar{I}} \eta_d^n \eta^{-(n+1)} = \frac{1}{1 + \eta_d \eta_o \bar{I} k_{\text{bl}}^{-1}} \left(1 + \frac{1}{\eta_d \eta_o \bar{I} k_{\text{bl}}^{-1}}\right)^{-n} \quad (7.38)$$

By introducing $N = \eta_d \eta_o \bar{I} k_{\text{bl}}^{-1}$ we can write this distribution for the number of detected photons n as

$$p(n; N) = \frac{1}{N} \left(1 + \frac{1}{N}\right)^{-(n+1)} \quad (7.39)$$

where N is the average number of photons detected $N = \sum_{n=0}^\infty n p(n)$.

In order to estimate the range of parameters in which the simplified model is valid, we compare it to the full model Eq. 7.34. Fig. 7.14 shows that the model works best, if k_{bl} is well separated from the blinking rates k and $1/T$. As mentioned in Sec. 7.2 and Sec. 7.3.6, this is indeed the case for the used experimental parameters.

The distribution of the intensity of a dimer (i.e. two fluorophores in a diffraction limited spot) $p_2(n; N)$ is obtained from the convolution of Eq. 7.39 with itself (121):

$$p_2(n; N) = \sum_{n'=0}^\infty p(n - n') p(n') \quad (7.40)$$

$$= \frac{1}{(1 + N)^2} \sum_{n'=0}^\infty \left(1 + \frac{1}{N}\right)^{-(n-n')} \Theta(n - n') \left(1 + \frac{1}{N}\right)^{-n'} \quad (7.41)$$

$$= \frac{1}{(1 + N)^2} \left(1 + \frac{1}{N}\right)^{-n} \sum_{n'=0}^n = \frac{n+1}{(1 + N)^2} \left(1 + \frac{1}{N}\right)^{-n} \quad (7.42)$$

Continued convolution with $p(n; N)$ (Eq. 7.39) gives the distribution for

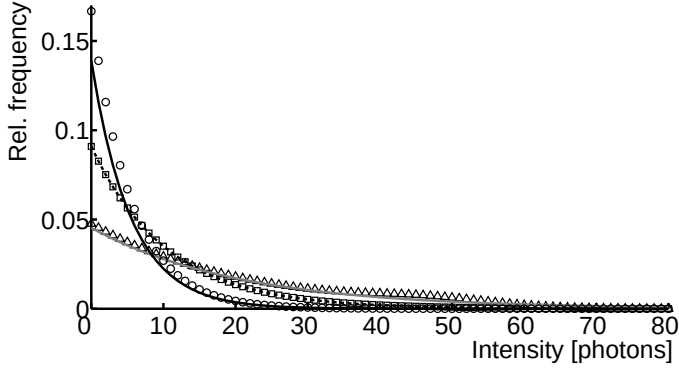


Figure 7.14

Comparison of the general model based on Eq. 7.34 to the simplified model Eq. 7.39 for long illumination times T . The open symbols correspond to the intensities calculated using the general model based on Eq. 7.34 with bleaching rate $k_{bl} = (0.05T)^{-1}$ (open circles), $k_{bl} = (0.1T)^{-1}$ (open squares), $k_{bl} = (0.2T)^{-1}$ (open circles). The switching rate is $k = (0.01T)^{-1}$ for all distributions. The lines correspond to intensities given by Eq. 7.39 with the same values for k_{bl} : $k_{bl} = (0.05T)^{-1}$ (black solid line), $k_{bl} = (0.1T)^{-1}$ (black dashed line), $k_{bl} = (0.2T)^{-1}$ (grey solid line)

higher multimers, like e.g. a trimer

$$p_3(n; N) = \sum_{n'=0}^{\infty} p(n - n')p_2(n') \quad (7.43)$$

$$= \frac{1}{(1 + N)^3} \left(1 + \frac{1}{N}\right)^{-n} \sum_{n'=0}^n (n' + 1) \quad (7.44)$$

$$= \frac{(n + 1) \left(\frac{n}{2} + 1\right)}{(1 + N)^3} \left(1 + \frac{1}{N}\right)^{-n} \quad (7.45)$$

In Fig. 7.2 the intensity distributions for a monomer, dimer and trimer with the same average number of detected photons N (per fluorophore) are compared.

7.C.5 Complete intensity distribution

By combination of the simplified model for the number of emitted photons Eq. 7.39 with the detection probability Eq. 7.25 a complete description of

experimental intensity distributions is obtained:

$$p(n, N) = \frac{C}{2} \left(1 + \operatorname{erf} \left(\frac{1}{\sqrt{2}} \left(\frac{n + \text{off}'}{2\sqrt{\pi}\sigma w} - t \right) \right) \right) \frac{1}{N} \left(1 + \frac{1}{N} \right)^{-(n+1)} \quad (7.46)$$

Since the signal width w is a property of the optics and the fluorophore and σ , the noise level, can be estimated from the data, N , the mean number of photons detected during the integration time, is the only free parameter. C is determined by normalization $\sum_n p(n, N) = 1$. C also gives the ratio between the number of actually detected single-molecule signals and the total number of single-molecule signals present: $C = \#(\text{detected signals})/\#(\text{all signals})$. Fig. 7.3C shows an example of the distribution for typical experimental values. Note that single-molecule intensity distributions have an intrinsic asymmetry due to the influence of the detection probability. As suggested in (223) there might be other experimental factors that can lead to asymmetric intensity distributions.

7.D Overlapping single-molecule signals

When two single-molecule signals are closer together than the diffraction limit $2 \cdot w$ ($= 2 \times$ signal width), they cannot be resolved anymore. Consequently, a dimer is observed. For a homogeneous distribution of molecules with surface density ρ , on average $\rho\pi w^2$ molecules are found in a circle of radius w . Those molecules would be observed as a single signal. Assuming a Poisson process for the positions of the molecules, the probability to observe an n -mer $p_{\text{cluster}}(n, w)$ at a signal width w is therefore:

$$p_{\text{cluster}}(n, w) = C \frac{(\rho\pi w^2)^n e^{-\rho\pi w^2}}{n!} \quad (7.47)$$

where C is determined by normalization: $\sum_{n=1}^{\infty} p_{\text{cluster}}(n, w) = 1$, so

$$p_{\text{cluster}}(n, w) = \frac{(\rho\pi w^2)^n e^{-\rho\pi w^2}}{(1 - e^{-\rho\pi w^2}) n!} \quad (7.48)$$

ρ is given by $N_{\text{mol}}/A_{\text{ROI}}$ where N_{mol} is the number of molecules in the region of interest (ROI) with area A_{ROI} . Note that this model is only valid if the density is far below the percolation limit.

The relation between the number of molecules N_{mol} and the number N_{signals} of observed signals is given by

$$N_{\text{signals}} = N_{\text{mol}} \left(\sum_{n=1}^{\infty} n p_{\text{cluster}}(n, w) \right)^{-1} \quad (7.49)$$

Fig. 7.15 shows the ratio of observed signals to the number of molecules $N_{\text{signals}}/N_{\text{mol}}$ for a typical experimental value of $w = 0.7 \text{ pxl}$, $\text{pxl} = 220 \text{ nm}$. Here, n -mers up to $n = 50$ are considered. For comparison, if only monomers and dimers are admitted (dashed line in Fig. 7.15), the amount of overlap is underestimated. For $\rho < 0.25/\text{pxl}^2$ both curves coincide, which means that for low densities, signals consist exclusively of monomers and dimers. In that regime, the ratio $N_{\text{signals}}/N_{\text{mol}}$ decreases linearly with density ρ . The monomer fraction α used in Sec. 7.3.5 is defined as $p_{\text{cluster}}(1, w)/N_{\text{signals}}$.

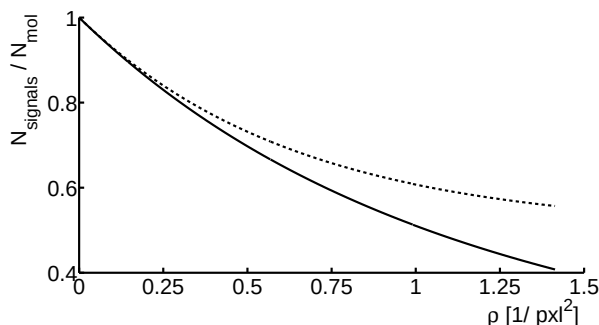


Figure 7.15

$N_{\text{signals}}/N_{\text{mol}}$ for $w = 0.7 \text{ pxl}$, $\text{pxl} = 220 \text{ nm}$. Solid line: n -mers up to $n=50$ are considered, dashed line: only monomers and dimers are considered

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SAMENVATTING

Het heterogene membraan - van lipide domeinen tot de effecten van kromming

Het celmembraan aan de buitenkant van elke cel is essentieel voor het leven. Het membraan is niet alleen een scheidingswand, die de cel in staat stelt om zijn eigen biochemische samenstelling te laten verschillen van die van de omgeving, maar ook een twee-dimensionale “reageerbuis” voor een groot aantal belangrijke reacties. Er zijn bijvoorbeeld bepaalde transmembraan eiwitten, zogenaamde receptoren, die een chemisch of fysisch signaal door het membraan naar het binnenste van de cel doorgeven. Dit gebeurt meestal door verandering van hun vorm. Receptoren geven het signaal door naar andere eiwitten die aan de binnenkant van het membraan zitten. Dit soort processen vinden plaats in nano - tot micrometer grote domeinen in het twee-dimensionale membraan. Lipide domeinen verschillen in samenstelling van de rest van het membraan. Het membraan is dus, in tegenstelling tot een gewone reageerbuis, niet homogeen maar bevat heterogeniteiten. De domeinen hebben een belangrijke functie: ze versnellen reacties door de reagentia dicht bij elkaar te brengen en zorgen ook voor specificiteit. Afhankelijk van hoe de (lokale) omgeving eruit ziet, kan activatie van dezelfde receptor verschillende veranderingen in het gedrag van de cel induceren. Kort gezegd is de nano- en microstructuur onlosmakelijk verbonden met de functies van het membraan en daarom een belangrijk onderwerp van onderzoek.

De experimentele studie van membraan heterogeniteit kan grofweg ingedeeld worden in twee verschillende manieren van aanpak. Aan de ene kant worden kunstmatige, gesimplificeerde membraansystemen als modellen gebruikt om fundamentele eigenschappen van het membraan geïsoleerd te bestuderen (hoofdstukken 2-4 van dit proefschrift). Aan de andere kant worden fluorescentie technieken met hoge resolutie in ruimte

en tijd toegepast om de invloed van de membraanstructuur op het gedrag van membraaneiwitten te bepalen (hoofdstukken 5-7 van dit proefschrift).

Hoofdstuk 2 beschrijft hoe de materiaaleigenschappen van kunstmatige membranen (in de vorm van “giant” vesikels), opgebouwd uit drie verschillende lipiden, nauwkeurig kunnen worden gemeten. Vesikels gemaakt van phospholipiden (met een laag smeltpunt), sphingolipiden (met een hoog smeltpunt) en cholesterol ontmengen spontaan beneden een kritieke temperatuur, die van de specifieke samenstelling afhangt. In een bepaald gebied van het fasediagram bestaat co-existentie van twee vloeibare fases, de “liquid ordered” (vloeibaar geordende) fase en de “liquid disordered” (vloeibaar ongeordende) fase. In analogie met fasescheiding in drie dimensies met een oppervlaktespanning tussen de twee fases, is er ook in een tweedimensionaal systeem een energie, die met de grootte van het grensvlak (oftewel grenslijn) groeit: de lijnspanning. De lijnspanning is een belangrijke parameter voor biofysische modellen, die het ontstaan en de stabiliteit van lipide domeinen in levende cellen beschrijven. Door het meten van thermische fluctuaties van de rand van het vesikel, en met hulp van een analytisch model voor de gemiddelde omtrek, konden we de lijnspanning tussen en de krommingsmoduli van de twee vloeibare fases bepalen. Bestaande biofysische modellen voorspellen dat de door ons gemeten lijnspanning (van ≈ 1 pN) in levende cellen tot lipide domeinen van maar zo’n 10nm zou leiden.

In **Hoofdstuk 3** hebben we vesikels met dezelfde lipide samenstelling gebruikt als in Hoofdstuk 2. In dit geval hebben we echter, voordat we het vesikel afkoelden om de lipiden te laten ontmengen, de osmotische druk aan de buitenkant verhoogd. Het resultaat van deze procedure is dat er een aantal kleine domeinen ontstaan die elkaar afstoten en daardoor tijdelijk stabiel zijn. Dit is in tegenstelling tot de vesikels uit Hoofdstuk 2, waar de fasescheiding volledig was. De afstotende kracht tussen de domeinen wordt veroorzaakt door de elasticiteit van het membraan en de vorm van de domeinen. De domeinen hebben een kromming die groter is dan de kromming van de hele vesikel; met andere woorden, de domeinen zijn “bulten” (“buds”) die boven het vlak van de andere fase uitsteken. Daardoor is er energie nodig om de domeinen dichter bij elkaar te brengen. Door de afstanden tussen domeinen te meten hebben we de grootte van deze membraan-gerelateerde wisselwerking bepaald en ook het effect op de verdeling van domein groottes gemeten. Met hulp van een kwantitatief model hebben we de stabiliteit van een systeem van “budded” domeinen

verklaard en de samenhang tussen de grootte van de wisselwerking en domein diameter bepaald. Onze resultaten hebben niet alleen betrekking op de fasescheiding van lipide systemen. Eiwitten die een kromming van het membraan veroorzaken wisselwerken in principe via dezelfde mechanismen.

Omdat we in onze experimenten zagen dat de domeinen niet homogeen op een vesikel verdeeld zijn, maar dat grote en kleine domeinen spontaan van elkaar gescheiden worden, hebben we in **Hoofdstuk 4** de invloed van de afstoting op de ruimtelijke verdeling van domeinen nader onderzocht. Omdat de grootte van de afstoting met de diameter van een domein toeneemt, verlaagt de scheiding van grote en kleine domeinen de totale energie van het hele systeem. De grote domeinen kunnen namelijk in dit geval meer ruimte innemen dan in een gemengd systeem. We hebben zowel in de experimenten, als in simulaties van dit systeem de grootte van een domein met die van zijn naaste burens vergeleken. In beide gevallen vonden we dezelfde correlatie: kleine domeinen hebben relatief kleine burens, en grote domeinen relatief grote burens. Omdat transmembraan eiwitten in principe op dezelfde manier wisselwerken als lipide domeinen, zouden membraan-gerelateerde wisselwerkingen ook voor de spontane organisatie van eiwitten kunnen zorgen.

Kunstmatige membranen kunnen altijd maar één specifieke eigenschap van het membraan in een levende cel modelleren. Daarom wordt in de tweede helft van dit proefschrift membraan heterogeniteit in levende cellen bestudeerd. In het bijzonder hebben we de observatie van enkele fluorescente moleculen (oftewel “single-molecule tracking”) toegepast om de invloed van domeinen op nano- of micrometer schaal te bepalen. Deze techniek is zeer geschikt vanwege zijn hoge ruimtelijke en temporale resolutie. De metingen van de beweging van enkele moleculen zijn echter soms moeilijk te analyseren, in het bijzonder als fluorescente eiwitten gebruikt worden. De fotofysica van deze moleculen is ingewikkeld, met name het feit dat fluorescente eiwitten tijdelijk “donker” kunnen worden (als het ware ‘aan’ en ‘uit’ gaan) en snel verbleken (waarna ze niet meer fluoresceren), maakt kwantitatieve metingen moeilijk.

In **Hoofdstuk 5** beschrijven we een nieuwe analysetechniek, “Particle Image Correlation Spectroscopy (PICS)”, die de problemen die door de fotofysica van moleculen veroorzaakt worden kan omzeilen. In plaats van een reconstructie van de door de ruimte afgelegde paden van individuele moleculen, de gebruikelijke methode in “single-molecule tracking”,

berekent PICS de correlatie in zowel ruimte als tijd tussen de posities van moleculen op twee opeenvolgende plaatjes in een serie van plaatjes. Uit de correlatiefunctie wordt de gemiddelde kwadratische afwijking (oftewel “mean squared displacement”, MSD) afgeleid. De MSD is karakteristiek voor de diffusie van een molecuul en weerspiegelt de microstructuur van het membraan.

In **Hoofdstuk 6** wordt PICS toegepast op de diffusie van de A_1 adenosine receptor, een “G protein coupled receptor” (GPCR). GPCRs zijn een belangrijk doelwit voor medicijnen omdat ze makkelijk bereikbaar zijn (aan de buitenkant van een cel) en een rol spelen in een groot aantal biologische processen. Een GPCR wordt geactiveerd door de wisselwerking met een klein molecuul (“ligand”) aan de buitenkant van het membraan. Door deze wisselwerking verandert de vorm van de receptor en wordt het voor hem mogelijk een wisselwerking met zijn G-eiwit aan te gaan. Vervolgens wordt het G-eiwit actief, geeft het signaal door aan andere moleculen en uiteindelijk aan de celkern. De wisselwerking tussen de GPCR en zijn G-eiwit gebeurt vrij snel na de activatie van de receptor. Deze observatie impliceert dat ofwel de receptor en het G-eiwit in hetzelfde kleine membraandomein zitten, of dat het G-eiwit en de receptor aan elkaar gekoppeld zijn nog voordat de receptor in aanraking met de ligand komt. Met behulp van PICS konden we vaststellen dat de diffusie van de receptor na activatie vertraagd is, maar dat ontkoppeling van het G-eiwit en de receptor tot versnelde diffusie leidt. Deze resultaten tonen aan dat de receptor aan zijn G-eiwit gekoppeld is, ook zonder wisselwerking met de ligand, en dat het G-eiwit verantwoordelijk is voor de wisselwerking met de membraanstructuur. Omdat de diffusie versneld wordt als het membraan van het onderliggende cytoskelet ontkoppeld wordt, gaan we ervan uit dat de waargenomen membraanstructuur aan het cytoskelet gerelateerd is.

Behalve door meting van diffusie kan membraan heterogeniteit verder nog op een andere manier waargenomen worden. Twee fluorescente moleculen die dicht bij elkaar ($\lesssim 200$ nm) zitten, bijvoorbeeld in een membraandomein, kunnen niet meer als individuele signalen waargenomen worden, maar geven één signaal met ongeveer de dubbele intensiteit. In principe kan zo door het meten van signaalintensiteiten het aantal moleculen in een domein worden bepaald. De fotofysica van de fluorescente eiwitten heeft echter opnieuw een grote invloed op de signaalintensiteiten en maakt de analyse moeilijk. In **Hoofdstuk 7** leggen we uit dat de verdeling van signaal intensiteiten meestal zo breed is dat het bijna onmo-

gelijk is om het verschil in intensiteit tussen een enkel molecuul en twee moleculen te zien. We laten echter ook zien dat, met de juiste keuze van experimentele parameters, het toch mogelijk maakt om individuele moleculen in levende cellen te tellen ondanks hun ingewikkelde fotofysica. We hebben de verdeling van intensiteiten uit de semi-klassieke Mandel theorie afgeleid en door experimenten bevestigd. Tenslotte hebben we de verdeling van een bepaald membraananker (van H-Ras) in membraan domeinen in levende cellen gekwantificeerd.

Samenvattend hebben we de membraan heterogeniteit in kunstmatige membranen en levende cellen bestudeerd. We hebben laten zien hoe beide manieren van aanpak tot complementaire inzichten in complexe biologische processen kunnen leiden.

PUBLICATIONS

1. "Dpp morphogen transcytosis studied with Particle Image Cross Correlation Spectroscopy (PICCS)"
S. Semrau*, L. Holtzer*, M. González-Gaitán, T. Schmidt
(* authors contributed equally)
in preparation
2. "Protein incorporation in giant unilamellar vesicles"
P.M. Shaklee*, S. Semrau*, M. Malkus, S. Kubick, M. Dogterom,
T. Schmidt (* authors contributed equally)
submitted to ChemBioChem
3. "Membrane heterogeneity - From lipid domains to curvature effects."
S. Semrau, T. Schmidt
Soft Matter, 5 (17), 3174-3186, (2009)
4. "Membrane mediated sorting"
T. Idema, S. Semrau, T. Schmidt, C. Storm
submitted to Phys. Rev. Lett.
5. "Membrane-mediated interactions measured using membrane domains"
S. Semrau*, T. Idema*, C. Storm, T. Schmidt
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Biophys. J., 96 (12), 4906-4915, (2009)
6. "Robust counting of autofluorescent proteins *in vivo*"
T. Meckel*, S. Semrau*, M. Schaaf, T. Schmidt
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submitted to Anal. Chem.

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CURRICULUM VITAE

Stefan Semrau was born on January 16, 1979 in Solingen, Germany. He studied physics at the Technical University Aachen, Germany from 1999 to 2005, specializing in theoretical solid state physics/many-particle theory and biophysics. In 2002 he became a student scholar of the German National Academic Foundation and received a diploma in physics in 2005. His diploma thesis was entitled "Electron transport through one-dimensional arrays of metallic nanoparticles". Semrau was awarded the Springorum medal of the RWTH Aachen for the diploma exams passed with distinction and he received the Schoeneborn award from the "Freunde und Foerderer der RWTH Aachen e.V." for outstanding achievement in physics. After obtaining his diploma Semrau joined the Physics of Life Processes group at Leiden University, The Netherlands as a PhD student. Under the guidance of Prof. Thomas Schmidt and co-supervision by Dr. Cornelis Storm he worked on material properties of membranes and single molecule tracking in living cells. The results of this work are presented in this thesis. In 2008 he was the Leiden Institute Of Physics' nominee for the "Discoverer of the year" of the faculty of science, together with Timon Idema. During his 4 year stay in Leiden Semrau presented his work at numerous summer schools and conferences in the Netherlands, Germany, the UK, France and the USA. He assisted with the lecture "Introduction to solid state physics", lectured "Introduction to quantum mechanics" and supervised several bachelor projects. In January 2010 Semrau will start a postdoctoral appointment in the Systems Biology lab of Alexander van Oudenaarden at M.I.T., USA.

