Summary

In the last decades, research in biology has developed towards progressively smaller scales and has now entered a nanoscale world where molecular interactions become visible, functioning of molecular motors can be measured, direction and speed of movement of molecules can be followed and protein structures are revealed. In parallel the required technologies, enabling to address, visualize and analyze molecules, follow the same trend of miniaturization. To investigate single molecules at ambient conditions, *in vitro* as well as *in vivo*, requires high resolution, molecular specificity and sensitivity. Near-field optics has the promise to fulfill these requirements. In this research we have developed a dedicated near-field scanning optical microscope (NSOM) for molecular biology and applied it to study the spatial organization of (fluorescently labeled) proteins at the cell surface. For the first time, protein clusters and individual molecules are resolved at the cell membrane with nanometer resolution using an optical method.

A short history of microscopy and its importance for cell biology is given in Chapter 1 of this thesis. Different types of fluorescence microscopy methodologies are described as well as their applicability for the investigation of biological specimens. In addition, an overview of available optical single molecule methods and their working principles is given. The spatial resolution of conventional optical microscopy methods is diffraction limited, allowing single molecule detection on an area with a low molecular density of only a few molecules per square micrometer. In addition, the relatively large depth of illumination (hundreds of nanometers) induces a large background contribution when studying cells. These problems are overcome by near-field optical techniques, where an exponentially decaying field illuminates the sample to a depth of below 100 nm. In NSOM a subwavelength aperture at the end of a sharp fiber probe is used as light source. In this way, the illumination volume is confined in three dimensions, resulting in the smallest possible illumination volume by optical means. The chapter is concluded with an overview of the human immune system, particularly focussing on the function of dendritic cells and T cells, placing the studied protein systems in a wider perspective.

Chapter 2 describes the technical details of the combined near-field/confocal scanning optical microscope. The microscope allows both near-field as well as a confocal type of illumination and has one sensitive detection path allowing single molecule detection in a polarization or wavelength dependent manner. The confocal part of the microscope is used to perform a quick pre-study of the sample and select an area for further in depth near-field investigation. In this chapter, the performance of the microscope is shown on the basis of measurements of fluorescently labeled molecules on cells, demonstrating three main advantages of NSOM over confocal microscopy in cell studies: 1) NSOM provides a high localization accuracy and spatial resolution, allowing to distinguish molecular entities in densely packed systems; 2) NSOM has a low penetration depth, avoiding intracellular autofluorescence; 3) NSOM provides simultaneous optical and topographical information. These advantages have been exploited in the following chapters for studying different types of proteins on the membrane of intact cells at the single molecular level.

In Chapter 3 the spatial organization of proteins (DC-SIGN) on the cell membrane of immature dendritic cells (imDCs) is investigated. These cells bind different types of pathogens such as viruses and bacteria, using DC-SIGN. We have investigated the spatial organization of DC-SIGN on the membrane as this is thought to be related to the pathogen binding capability of the cell. Near-field optical images of DC-SIGN proteins, labeled via antibodies with Cy5, show a full coverage of the membrane with individual fluorescent spots of variable intensities. Amongst the large number of spots, only a few show single molecule emission. The typical single molecule intensity of Cy5 molecules is measured and used to relate the intensity of each fluorescent spot to the number of Cy5 molecules present at that location. The spots contain from 1 to over 200 Cy5 molecules and more than 80 % of all spots are molecular domains. Despite the large variety in molecular content, the distribution of measured domain sizes is rather narrow and peaks at 200 nm. Correlation of the domain sizes with the number of molecules in each domain reveals a large variety in molecular density. Assuming an average labeling efficiency of 3.5 Cy5 molecules per antibody - which is explicitly measured in Chapter 5 - and a one to one ratio between antibody and protein, the average nearest neighbor distance (*nnd*) between DC-SIGN proteins within a domain is estimated to be 39 nm. Distances between the domains indicate a random domain distribution pattern. Thus, we have recognized a two-layer hierarchical organization of DC-SIGN spacing, where DC-SIGN resides in domains (layer 1), which are randomly spread (layer 2). As DC-SIGN binds to various pathogens with different affinities, we hypothesize that 1) DC-SIGN makes use of variable molecular densities to allow binding to different types of pathogens; 2) DC-SIGN domains are randomly distributed to maximize the probability of

hitting a pathogen in an accidental encounter of a cell and a pathogen.

Clustering of membrane molecules into domains, such as found in Chapter 3, is a common phenomenon in cell biology and known to facilitate specific cell functions. In chapter 4 a simple, two-dimensional model is introduced to explain the advantages of clustering and investigate the importance of domain size and molecular packing density in a domain. The model describes the probability for an object of certain size hitting a minimum number of receptor molecules on the cell membrane in an accidental object-cell encounter. We have introduced Monte Carlo simulations, enabling the use of distributions of domain properties. We have performed simulations using realistic numbers for the domain properties and investigated the binding capabilities of the immature dendritic cell. Using a constant total number of surface molecules and various types of spatial organization, the simulations show that 1) domains are beneficial over a random spread of individual molecules if more than a specific number of hits is needed in an encounter to obtain a stable object-cell bond; 2) domain properties are particularly important for the hit probability if the object size is comparable to the domain size; 3) the origin of molecular packing density, given either by domain size or domain content, is important to achieve a high hit probability if the object is larger than the domain; 4) an optimum exists for the spatial distribution of receptor molecules to bind objects of specific size with a specific number of receptors in the object-cell contact area. Simulations including distributions of the domain properties of DC-SIGN show that the wide spread in domain content ensures a larger number of hit receptors compared to an area with domains having one average size and content. Moreover, the spatial organization of DC-SIGN seems to be optimized for small virus-like objects.

In Chapter 5, the sudden termination of single molecule fluorescence, i.e. discrete photobleaching, is used as a specific time dependent single molecule property to count the number of fluorophores and localize fluorophores within densely packed areas. The labeling efficiency of Cy5-labeled antibodies was determined by recording the fluorescence emission from the densely packed Cy5 fluorophores in time and counting the number of discrete photobleaching steps. Similar time dependent studies on densely packed molecular domains show first a bulk-like exponential decay of the fluorescence intensity. Because the decrease in intensity is accompanied by a decrease in shot noise, discrete photobleaching steps of the remaining fluorophores can be counted when the shot noise falls below the signal level of a single molecule. Photobleaching is further exploited by counting and localizing single molecules in an area of densely packed fluorophores. Using NSOM, the area is sequentially imaged. As the fluorophore density decreases in each image, single fluorophores become gradually visible, which enables to count them as well as to pinpoint them individually. This method reveals the real spatial

distribution of fluorophores and unravels the molecular composition of domains.

In Chapter 6, the spatial organization of membrane proteins on T cells is investigated. The proteins of interest are IL-2Rα and IL-15Rα, subunits of the Interleukin receptor trimers IL-2R and IL-15R, respectively. These receptors perform a distinct function for the cell from the DC-SIGN proteins studied in Chapter 3. IL-2R and IL-15R bind specifically to Interleukins IL-2 and IL-15, respectively. Upon binding, signals are transmitted to the T cell, stimulating cell growth, cell differentiation and cell death. Interestingly, although both receptors are structurally similar, sometimes they seem to have contrasting effects on the course of life of a T cell. We have studied the α-subunits because they are thought to be essential for these contrasting effects. Near-field fluorescence images of Cy5-labeled IL-2Rα and IL-15Rα show domains as well as individual entities on the cell surface. Also single Cy5 molecules are visible, allowing to build a single molecule intensity distribution which is used to relate the intensity to the number of present Cy5 molecules. For both IL-2Rα and IL-15Rα, the intensity distributions of all fluorescent spots show two populations, i.e. one consisting of single proteins and the other of protein domains. This quantitative analysis also reveals the number of molecules residing within and outside domains. The domains of IL-2Rα and IL-15Rα are similar in size having a rather constant diameter of about 400 nm. Dual color near-field experiments show that IL-2Rα and IL-15Rα co-localize on the membrane, which is thought to be essential for the signaling capability of both IL-2R and IL-15R. A fundamental difference between the IL-2Rα/IL-15Rα and DC-SIGN domains is found when correlating domain size with number of proteins within the domain. Namely, both IL-2Rα and IL-15Rα show a constant molecular packing density for all domains of 1350 IL-2Rα/μm^2 and 120 IL-15Rα/μm^2, respectively. This difference in spatial organization for adhesion and signaling molecules indicates the close structure-function relation between spatial membrane organization and cell function.

This thesis shows the value of near-field scanning optical microscopy combined with single molecule sensitivity for cellular and molecular biology. In addition, it shows the possible ways to exploit this technique for revealing the nanometric organization of molecules on the cell membrane.