Shedding light on endocytosis with optimized super-resolution microscopy

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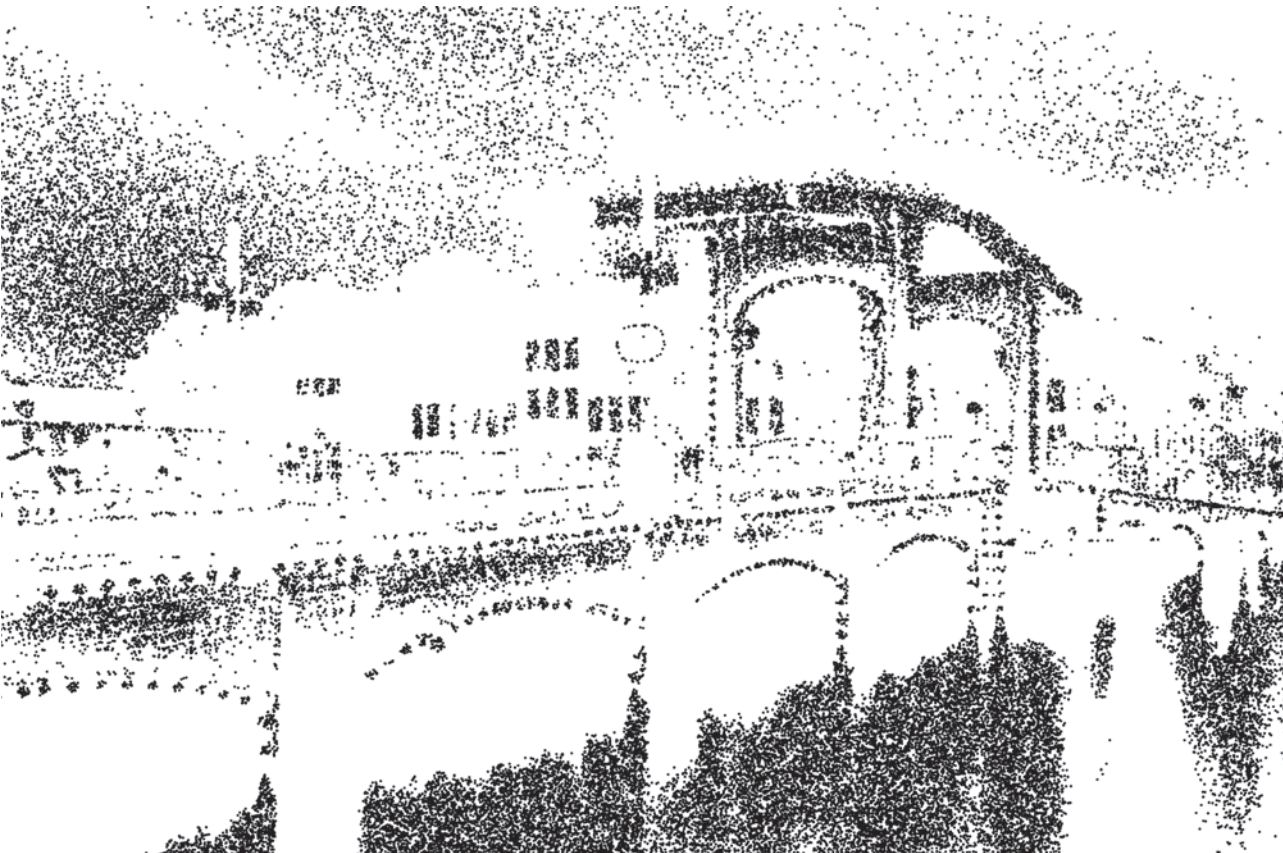
Chapter 6

Summarizing discussion



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The advent of super-resolution microscopy made it possible to surpass the diffraction limit of conventional optical microscopy. The increase in resolution that super-resolution microscopy enabled, like many of the breakthroughs in the history of science, did not happen through small continuous improvements but in one big step. From the 50s to the 80s a few concepts to improve the resolution in far-field optical microscopy were proposed, but they were not applicable or represented only a slight improvement in resolution [72]. The leap came with the proposal of the use of fluorophore transitions in the early 90s with the concepts of STED and GSD [226,227]. About ten years later, methods that proposed related types of on-off transitions for switching of fluorophores, known as stochastic single molecule resolution microscopy (SMLM), were published [74-77]. The application of super-resolution microscopy has yielded a remarkable amount of publications, some with major impact in the understanding of cellular structures. Yet, as a relatively new technique, super-resolution microscopy is still under development and many aspects of the process of obtaining a super-resolution image can be further improved. In this thesis, we focused on the further development of stochastic SMLM and its application.

Good riddance: structured background subtraction.

In this thesis we first focused on the correction of suboptimal dye blinking. Blinking of fluorescent molecules is the basis of stochastic SMLM. Blinking properties, in particular photon yield and on-off duty cycle, are crucial for the final image outcome and vary among dyes. The brightness of a blink will determine how accurate its center can be localized. The duty cycle will determine the fraction of time a fluorophore spends in the on state [78] and therefore how many fluorophores are on at the same time [78,81,82]. In samples labeled using fluorophores with high duty cycles a relatively high structured background intensity is usually present, caused by neighboring fluorophores that are on for prolonged times. Structured background (also called sample background [80] substantially reduces the localization precision and leads to a commonly seen image artifact. In **Chapter 2** we show that structured background can be corrected in the original blinking movie, simply by calculating the median brightness per pixel over a window in time and subtracting it. Because bright and short “on” events clearly stand out, they are spared. This easy trick leads to blinking movies that contain only bright and short events that can be localized with higher precision. As a proof of principle, we use samples simultaneously labeled with the best known blinking dye, AlexaFluor647, and a commonly used dye that has high duty cycle in the same imaging buffer, AlexaFluor532. We show that application of a temporal median filter to AlexaFluor532 blinking movies corrects the sample background yielding images with similar quality as the ones obtained with AlexaFluor647. Furthermore, we show that this trick can also be applied to blinking movies of fluorescent proteins, which commonly have high duty cycles and are not too bright, to obtain remarkable improvements in image quality. Importantly, application of a temporal median filter expands the list of fluorophores that can be used for stochastic SMLM, which increases the possibilities for multicolor imaging.

Preserving the sample. Fixation of actin and its binding proteins.

Because of the importance of blinking, several reviews and studies have focused on fluorophore switching, and many localization algorithms have been developed that aim to improve final image quality [78,127]. But even with blinking and localization optimized, the final SR image can only be as good as the preparation: image quality ultimately depends on the fidelity with which the sample is preserved during the fixation process. This aspect, which

has been well-studied in the electron microscopy field, has only recently been addressed for super-resolution microscopy [85,86].
In **Chapter 3** we focus on the optimization of the preservation of samples of the actin cytoskeleton and its binding proteins for super-resolution microscopy studies. We show that glutaraldehyde fixation procedures, which necessitate pre-extracting of the samples due to its low permeability, can result in loss of weakly bound or soluble proteins, such as mDia1 and WAVE2. Moreover, it is well known that glutaraldehyde can induce (small) changes in the tertiary structure of proteins, hindering epitope sites for antibodies and making immunofluorescence labeling ineffective. We show, using a commonly used anti-clathrin heavy chain (CHC) antibody, that the epitope hindering effect of glutaraldehyde detected in super-resolution images is not always obvious at lower resolution, making confocal or widefield microscopy inspection useless.

Of course, some of the adverse effects of glutaraldehyde were known to microscopy users. However, glutaraldehyde fixation preserves actin fibers better than any other procedure. How to image actin and its partners then, using fluorescence microscopy at high resolution? We show that with careful consideration of the fixation parameters, e.g. temperature, time and pH, during the fixation procedure, paraformaldehyde fixation can yield high quality super-resolution images of actin, while preserving weak binding proteins. Moreover, besides maintaining the fine actin structure, our fixation protocol is readily compatible with immunolabeling, enabling artifact free multicolor super-resolution imaging of actin and accessory proteins. Because super-resolution microscopy is a recent development, the field lacks extensive controls and experience, and therefore careful optimization of the fixation procedure is of upmost importance when new proteins are to be studied. To make a first step in the direction of specific sample preparation guidelines for super-resolution, we deposited a detailed protocol and troubleshooting table in Protocol Exchange.

Where are we and where are we going.

In this thesis we worked on optimizing sample preparation and post imaging background correction for super-resolution microscopy. Of course the quality of super-resolution microscopy experiments is determined by the sum of many parameters, each of which is under constant development. During the preparation of this thesis, several new developments for the improvement of super-resolution microscopy were published, including the improvement of photo-switching buffers to enhance blinking [84,228], buffers that are compatible with one or more dyes [83], fast switching brighter fluorescent labels [229-231], faster and/or more sensitive cameras [232], the introduction of a lens that made 3D imaging possible and remarkably easy [126], and so on. Finally, probably the most prolific area of developments for super-resolution microscopy has been the localization methods field, with several different mathematical approaches to calculate the center of blinks and several pre and post analysis tools, published in the last years (to many a few [108,110,137,147,233]).

An important aspect is the size of the used labels. In a final super-resolution image, localizations are not at the center of the labeled molecules, but at the centers of the fluorophores that label them. These centers may be displaced with respect to the real position of the molecules. The displacement will vary depending on the type of labeling used. Fluorescent proteins are small 4 nm long barrels that in general are directly attached to the molecule of interest and therefore do not induce a large displacement. Also, organic fluorophores with their small size of about I nm are not a big concern. However, the linker

moiety that is used for attaching the fluorophore to the molecule can be large, and this generally causes large displacements. Antibodies, the most commonly used linkers, are about 8-10 nm long and when a combination of primary and secondary antibody to label a molecule is used, this may induce a displacement of up to 20 nm [80]. Many efforts have been put into developing smaller linkers between fluorophore and molecule. For example, nanobodies [234] and aptamers [235] are smaller options that bind the targeted molecules with high specificity while already reducing the displacement significantly. Other strategies to label molecules with very small linkers are Snap-tag [236], HaloTag [237], and click-chemistry [238]. In essence, these are protein domains used to attach chemical fluorophores with high affinity and specificity but these approaches can induce high background and they require genetical modification of the molecule of interest.

Besides smaller linkers is also needed to design fluorophores that blink optimally for SMLM. That is, molecules that can yield a large number of photons in short times, improving the possibilities for live cell super-resolution microscopy studies; molecules that are independent of the environment for their blinking, such as fluorescent proteins in which the fluorophore is protected by the barrel; or that are linked to a reducing agent, and so on [72,127].

"There are no right answers to wrong questions". What to use super-resolution microscopy for.

To obtain relevant information with SR microscopy, it is essential to define which questions it can answer. Stochastic SMLM should not be the first choice to study protein dynamics. Although some studies have used this type of super-resolution in live cells, there is a drawback implicit in the concept of super-resolution microscopy that makes live-cell studies challenging. The trick of switching fluorophores on and off is essential for surpassing the resolution limit, but it makes the imaging procedure very slow. Localization of fluorophores is impaired by movement of proteins and structures in living samples, and creation of the final SR image takes time. New faster CMOS cameras make the process of imaging shorter but often at the cost of photon collection, and therefore resolution is sacrificed [232]. Fluorescent proteins are not as bright as small organic dyes, but the latter may be toxic for cells and usually are not too specific, yielding high background. Importantly, inducing photoswitching requires the use of high laser powers that cause phototoxicity [239].To overcome the challenge, bright fast-switching fluorescent proteins are still in development, and in the future, in combination with fast cameras will possibly make live-cell super-resolution studies possible.

Super-resolution microscopy should not be the choice for the study of large cellular structures. On the other hand, for very small structures, the still superior resolution of electron microscopy is much more suited. However, at an intermediate scale, there are many advantages in using super-resolution microscopy instead of electron microscopy. Firstly, electron microscopy studies require tedious and long sample preparation and acquisition is slow, typically yielding only a limited set of images. Super-resolution microscopy sample preparation is more accessible, and can be carried out at high throughput. Super-resolution microscopy is based on fluorescence microscopy and therefore molecules or structures of interest can be screened for and selected "by eye" before collecting the high-resolution image. Moreover, it is important that several different molecules can be imaged in the same cell. In electron microscopy, with immune-gold labeling, only two "colors" (small and large gold particles) can be discriminated. In super-resolution microscopy at least one more color can be added and large efforts are being made to separate emission of fluorescent dyes

to allow more than three color imaging. Even so, already in two color studies, the labeling density that can be achieved with super-resolution is still much higher than the one that can be achieved in electron microscopy. A very promising approach is the combination of super-resolution microscopy and electron microscopy, which allows the fluorescent tagging of molecules imaged at high resolution, which can later be resolved at Angstrom resolution. A few recent studies have shown the advantages of such promising combination [240-242].

Characterizing the intrinsic clustering component.

One application of quantitative super-resolution microscopy is the study of protein clustering, for which several methods are currently applied. The organization of entities in clusters, like plants or trees on a field, has been the subject of study for many years, and several methods to describe and quantify these organizations have been developed. Because of the achievable resolution, super-resolution microscopy prompted a series of studies revisiting membrane protein clusters and membrane composition organization, a topic long discussed in cell biology. Initially, several previously reported methods for analysis of clusters were directly applied, such as Ripley's K [222,243,244]. However, as explained earlier on in this thesis, in stochastic SMLM every protein is localized multiple times due to, first, the presence of multiple labels per antibody and secondly, multiple blinking of each label, a phenomenon we call intrinsic clustering of localizations.

In **Chapter 5** we use a method that has already been employed for the analysis of protein clusters on cell membranes and adapt it to better describe our data with intrinsic clustering. To better analyze clusters in super-resolution data, Sengupta and colleagues [103] developed a model that aims to factor out the intrinsic clustering component in pair correlation analysis of super-resolution data. However, in this model, Sengupta and colleagues assume that intrinsic clustering adds a Gaussian distribution term to the analysis. In our experimental data, we found that the cluster formed by localizations of a single antibody is Cauchy distributed. A similar observation was also reported in an unrelated study [245]. Furthermore, we show that using the wrong assumption on the distribution may lead to overestimation of protein clustering. Unlike the clustering methods used in many published SR studies that ignore intrinsic clustering, our modified model is especially suited for cluster analysis of stochastic SMLM data.

Helping understand endocytosis with super-resolution microscopy.

In this thesis, in **Chapters 4 and 5** we apply our improved super-resolution microscopy to study the role of actin in clathrin mediated endocytosis (CME) and the state in which the epidermal growth factor receptor (EGFR) exists on the plasma membrane of different cells. Endocytosis controls the composition of lipids and proteins of the plasma membrane, and thus the manner in which cells respond to their environment. Endocytosis is a well-orchestrated process, and many proteins with different functions contribute at different points in time. Given the dynamic nature and small size of endocytic structures, a combination of normal resolution live cell microscopy and super resolution fixed cell microscopy provides the best tools to understanding how they are organized in space and time.

In **Chapter 4** we study clathrin mediated endocytosis, the endocytosis process that uses clathrin as a coat protein to cover endocytic vesicles. Using super-resolution microscopy we explore the difference between flat clathrin plaques (FCP) and clathrin coated pits (CCP)

and show that FCPs function as hubs for the formation of dynamic endocytic clathrin coated vesicle. Moreover, we show that the formation of these vesicles is controlled by N-WASP and Arp2/3 complex mediated actin polymerization. In this study we also map, for the first time, the N-WASP domains involved in the differential recruitment of the protein to CCPs and FCPs.

We found that actin controlled FCPs recruit the membrane receptors EGFR and LPAR1, and that they can be crucial in the regulation of signaling through these receptors. In cells in which actin polymerization was disrupted by means of N-WASP knockdown, internalization of both LPAR1 and EGFR was impaired. However, we found that only upon addition of LPA, and not EGF, stimulation of Akt signaling was enhanced, in an LPAR1-dependent manner.

Are flat clathrin plaques signaling platforms?

The proteins that constitute a signaling pathway need to come in close proximity in order to interact. Their localization and availability is crucial for the signal propagation and intensity [246]. Activated receptors and their signaling molecules may be sequestered together in clathrin coated structures (CCS). Recent studies have proposed that CCS are not only endocytic domains but also signaling platforms that are specialized in different pathways [247,248]. Garay and colleagues [248] proposed that clathrin scaffolds are plasma membrane signaling microdomains required for certain signaling receptors. They showed that upon genetic and biochemical ablation of clathrin, the signaling of Akt was reduced upon stimulation with EGF. Because Akt signal was not affected when endocytosis was blocked by other means, such as inhibition of Dynamin II , they concluded that clathrin itself has a direct role in regulating Akt activation via EGFR. Such regulation of Akt by clathrin was independent of its endoctytic role. Along the same lines, Eichel and colleagues [247] showed that upon triggering of the B1AR receptor, activated β-Arrestin dissociates from the receptor and travels to CCS where it signals so as to activate MAPK, until vesicle scission occurs. Although β-Arrestin is known to activate MAPK signaling from endosomes [1], this study shows for the first that it can also signal to activate MAPK from CCS.

Our results also indicate that in actin-controlled FCPs, specialized LPAR1 to Akt signal transduction takes place. It has been shown using PIP2 probes [173] that there is a high concentration of PIP2 in sites of CCV formation that diminishes after fission [246]. For the Akt pathway to be activated, activated PI3K needs to come in proximity with its substrate and in the same way PDKI and Akt need to be recruited together to specific sites [246]. In fact, CCSs can exhibit PI3K activity [249]. When endocytosis is slowed down due to reduced actin polymerization, this can give these proteins more time to interact in the signaling platforms, thereby strengthening the signal.

The view that CCS may function as signaling platforms is very recent. Much work needs to be done to understand how the different signaling CCS are regulated and what drives specific localization of different signaling platforms to different CCS.

Inducing endocytosis of overexpressed EGFR with Perifosine.

Aberrant receptor signaling is common in many types of cancer. A common clinical approach for regulating aberrant receptor signaling is the induction of its endocytosis. In **Chapter 5** we investigate how Perifosine, a synthetic lipid drug, regulates EGFR signaling.

Perifosine induces the endocytosis of EGFR in a non-conventional mechanism that doesn't depend on its activation. The endocytosed EGFR is not phosphorylated and it also doesn't get ubiquitinated. However, Perifosine only exerts this effect in cells that overexpress EGFR. To investigate possible differences between cells that do and do not overexpress EGFR, we used our improved clustering analysis method. We show that EGFR is pre-clustered in cells with high expression and not in cells with low expression, even in the resting state. While these results are preliminary, we hypothesize that the presence of pre-clustered EGFR is a pre-requisite for Perifosine to exert it's effect. One possibility is that Perifosine, as a non-natural lipid, disrupts the physicochemical properties of the membrane thereby inducing the endocytosis of EGFR. Stimulation of endocytosis and degradation of ErbBs is an attractive idea to inhibit tumor growth, especially in the cases in which the receptors are overexpressed.

In summary, in this thesis we have contributed towards the further development of important aspects of super-resolution microscopy and we have applied improved super-resolution microscopy to study the regulation of signaling and endocytosis of important membrane receptors.