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.................................
Exploring the Limits of Single Molecule Localization Microscopy using Realistic Simulations with a Focus on Presynaptic Nerve Terminals

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Abstract

Single molecule localization microscopy (SMLM) has been established as a powerful technique to investigate biological samples with a resolution well below the diffraction limit of light microscopy. However, the limits of these new techniques have not yet been probed. In particular, the localization of multiple targets using different fluorescent dyes is difficult due to chromatic aberrations. Hence, the aim of this project was to build a microscope based on the principle of stochastic optical reconstruction microscopy (STORM), which can be used to simultaneously acquire 3D aberration-free images of two fluorophore-labeled structures, establish an automated imaging workflow and systematically probe the possibilities and limits of SMLM.

The microscope was built and used to record images of F-actin and synaptophysin, a protein enriched at synaptic vesicles, in the rat’s calyx of Held, a glutamatergic model synapse. Contrary to our expectations, the experiment resolved neither actin filaments nor clearly distinct synaptic vesicles. To validate the experimental results and to define the limits of SMLM more precisely, a realistic simulation tool for SMLM experiments called SuReSim (super-resolution simulation) was developed. In SuReSim, 3D models of ground truth structures (e.g. filaments or organelles) can be imported and the expected outcome of a SMLM experiment can be simulated taking into consideration a multitude of parameters. Two options for the resulting output are available: either a list of simulated localizations, compatible with SMLM-specific 3D viewers or a realistic Tiff stack resembling raw data as recorded during a SMLM measurement. SuReSim was used to simulate realistic F-actin and synaptophysin imaging results based on model structures derived from electron microscopy. The simulations confirmed the experimental results, no distinct structures could be resolved. Additional simulations were performed on other biological samples to find the limitations of SMLM microscopy. The packing density of the structure, the density of binding sites, the localization precision, the labeling efficiency and the label length were identified as limiting factors for SMLM measurements.

Due to the many factors contributing to a SMLM measurement, it is not possible to find universal limits, but only certain sets of parameters that are necessary for a successful experiment. These limits have to be identified individually for each target structure. SuReSim can help to make the decision, whether or not SMLM is the right tool to address a specific research question.
Zusammenfassung

Einzelmolekül-Lokalisations-Mikroskopie (SMLM) hat sich als wichtiges Werkzeug durchgesetzt um biologische Proben mit Auflösungen weit jenseits der durch Beugungseffekte verursachten Auflösungsgrenze der Lichtmikroskopie zu untersuchen. Allerdings sind die Schranken dieser neuen Methoden noch nicht hinreichend ausgelotet worden. Insbesondere die Darstellung mehrerer Proteine durch den Einsatz verschiedener Farbstoffe stellt sich aufgrund der chromatischen Aberration als schwierig heraus. Das Ziel meiner Arbeit war daher der Aufbau eines Mikroskops für direkte stochastische optische Rekonstruktionsmikroskopie (dSTORM), welches dreidimensionale Aufnahmen von zwei Fluorophor-markierten Strukturen ohne chromatische Aberration ermöglicht, die Entwicklung eines automatisierten Arbeitsablaufs bei der Bildaufnahme und Bildanalyse sowie die systematische Erfassung der Möglichkeiten und Limitierungen der SMLM.


Zusammenfassung
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List of Publications

Reviewed Papers:


* shared first authorship

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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2D</td>
<td>Two Dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three Dimensional</td>
</tr>
<tr>
<td>API</td>
<td>Application Programming Interface</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary Units</td>
</tr>
<tr>
<td>BG</td>
<td>Benzylguanine</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer-Aided Design</td>
</tr>
<tr>
<td>(C)AZ</td>
<td>(Cytomatrix of the) Active Zone</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>EMCCD</td>
<td>Electron Multiplying Charge-Coupled Device</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous Actin</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>GA</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GUI</td>
<td>Graphical User Interface</td>
</tr>
<tr>
<td>H</td>
<td>Hour</td>
</tr>
<tr>
<td>HILO</td>
<td>Highly Inclined and Laminated Optical Sheet</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IR</td>
<td>Infra-Red</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>kW</td>
<td>Kilo Watt</td>
</tr>
<tr>
<td>MEA</td>
<td>Mercaptoethyamine</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>NeNA</td>
<td>Nearest Neighbor in Adjacent Frames</td>
</tr>
<tr>
<td>Ni-trisNTA</td>
<td>Tris-N-Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>ns</td>
<td>Nanosecond</td>
</tr>
<tr>
<td>PALM</td>
<td>Photoactivated Localization Microscopy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PSF</td>
<td>Point Spread Function</td>
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<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SMLM</td>
<td>Single Molecule Localization Microscopy</td>
</tr>
<tr>
<td>SNARe</td>
<td>Soluble NSF Attachment Protein Receptor</td>
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<tr>
<td>SRM</td>
<td>Super-Resolution Microscopy</td>
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<tr>
<td>SSIM</td>
<td>Saturated Structured Illumination Microscopy</td>
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<tr>
<td>STORM</td>
<td>Stochastic Optical Reconstruction Microscopy</td>
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<tr>
<td>SuReSim</td>
<td>Super-resolution Simulation</td>
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<tr>
<td>SV</td>
<td>Synaptic Vesicle</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>Tiff</td>
<td>Tagged Image File Format</td>
</tr>
<tr>
<td>TIR</td>
<td>Total Internal Reflection</td>
</tr>
<tr>
<td>TOM20</td>
<td>Translocase of Outer Membrane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
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1 Introduction

Fundamental processes of life often happen on a very small scale. Many of these processes are performed by molecular machines, complexes predominantly built from different proteins with specialized functions. Examples are motor proteins like myosin or kinesin or the ribosomes that play an essential part in protein synthesis. This list can easily be extended to include proteasomes, important for the regulation of protein levels within a cell or the SNARE (soluble N-ethylmaleimide-sensitive-factor attachment receptor) complex, essential for synaptic vesicle docking and Calcium-dependent fusion, thereby underlying signal transmission in chemical synapses.

Beside protein composition of molecular machines, the protein distribution, especially the attachment between different proteins, is essential for biological processes. One important question concerns the distribution of actin at the active zone of synapses and the role arising from the particulars of its organization in orchestrating the synaptic vesicle cluster and synaptic vesicle replenishment at in the presynaptic nerve terminal. For the functionality of the above mentioned protein complexes the exact spatial arrangement of the individual proteins is essential. The size of these complexes is usually too small to be investigated using conventional fluorescent microscopy. Because of that, electron microscopy (EM) was used to study these protein arrangements.

The invention of super-resolution microscopy (SRM) ten years ago provided a new set of tools to explore biological structures on the scale of tens of nanometers. Although electron microscopy yields better resolutions than all super-resolution techniques, it bears its own challenges. For example, EM images show different shades of electron density in high resolution, but provide no information about molecular identities. To identify specific proteins EM users conventionally employ gold particles attached to antibodies, forced to accept several restrictions. First, the penetration depth of the antibodies is limited. The result is a high concentration of the label in the exposed regions and only few labels on the inside of the sample. This problem is even more acute in densely packed structures like the active zone. Second, labels can unspecifically bind to unrelated protein structures or organelles, such as mitochondria. Third, stainings against multiple proteins with differentiable labels are difficult using EM. One way to differentiate between labels is the usage of gold particles of different size. But with this technique it is difficult to achieve more than two distinguishable labels.

In contrast to EM, super-resolution microscopy uses fluorescent dyes as markers. The size of fluorophores usually is in the range of nanometers. Combined with small labels like nanobodies or phalloidin, the size of the label-fluorophore complex is much smaller than the size of immunogold, resulting in a better tissue penetration and label epitope distance. In conventional fluorescent microscopy, spectrally distinct fluorophores are used to record multiple structures at once. This leads to chromatic aberration. To circumvent chromatic aberrations a technique called spectral demixing was developed. Using spectral demixing it is possible to record signals from multiple different fluorophores in one measurement without aberrations. The number of labeled proteins can be further increased if new structures are restained or reversibly binding labels are used. Another advantage of super-resolution techniques is that they can also provide 3D information of the imaged structures. Additionally, the sample preparation for super-resolution microscopy is faster and easier than the preparations needed for EM.
Introduction

In summary, super-resolution techniques lack the resolution of electron microscopy but still reach resolutions that are sufficient to study the molecular composition of many biological systems. The labeling approaches used for light microscopy are smaller, more flexible and better suited to yield higher penetration depths than labels used for electron microscopy. Being a relatively recent imaging method the limits of super-resolution imaging techniques are not yet fully understood, especially when applied to questions in the field of neuroscience. Especially for SMLM the image formation is indirect and requires an additional reconstruction step.

In this chapter the fundamental principles of microscopy and super-resolution microscopy will be introduced. The first section will introduce fluorophores and labels, which play an essential role for fluorescence microscopy. It is followed by a summary of different microscopy techniques with a special focus on single molecule localization microscopy. After that, a brief introduction to the biological test systems used in this thesis will be given. The last section will briefly explain the signal transmission and the active zone of chemical synapses.

1.1 Labels

All fluorescent microscopy techniques require fluorescent samples. The main techniques to create fluorescent samples are either to stain the structures of interest or to let the cell endogenously express the proteins of interest with fluorescent proteins directly fused to them.

In the case of fluorescent stainings, important properties of the labels are their size and specificity. The labels must be able to reach a binding site in order to bind to it. The penetration of the sample gets better with smaller label sizes, especially in densely packed samples like tissue. The label should bind only to the protein it is intended to, which is called specificity of the label.

1.1.1 Immunohistochemistry

Antibodies are used by the immune system to specifically bind to pathogens like viruses and bacteria. In research applications antibodies are often used to attach fluorophores to the protein of interest. Using antibodies to detect antigens in cell or tissue sections is called immunohistochemistry (IHC). It was first introduced by Albert Coons in 1941.

A conventional antibody is a large, Y-shaped protein consisting of two larger (or heavy) and two smaller (or light) chains. There is a constant part and a unique part at the tips of the arms of the antibody. An unique molecule of the pathogen, called antigen is recognized via the Fab’s variable region. The Fab region contains a paratope, consisting of the paired N-terminal variable domains of the heavy and light chains, a small region of five to ten amino acids, which is specific for one particular epitope of the antigen. Emil Fischer described this binding mechanism as Lock-and-Key model. The base of the human antibody is called the Fc region or Fc fragment and consists of a part of the heavy chain. In mammals antibodies are secreted from B cells. There are monoclonal antibodies which only bind to a single epitope of an antigen. In contrast, polyclonal antibodies bind to multiple epitopes of an antigen. To tag a certain protein, antibodies with antigen binding regions complementary to the epitope of the target-protein are used.

There are two ways to label the proteins of interest with a fluorescent marker, an indirect and a direct method. For the indirect labeling method two antibodies are used. The so-called primary antibody is used to recognize the antigen of interest and it contains species-specific antigens. Antibodies from different species can be used to provide unique epitopes. The fluorophore is conjugated to the secondary antibody which recognizes and binds the unique epitopes of the primary antibody. In the second method a fluorophore is directly conjugated to the primary antibody. While the used of primary and secondary antibodies gives more flexibility for the
experimental design it also increases the distance between the epitope of interest and the fluorescent marker.

![Figure 1-1 Scheme of an antibody. The antibody consists of two longer, heavy chains and two shorter, light chains. The antigen binding sites are formed from both the heavy and light chains and recognize the antigens specifically.](image1)

IHC exploits the specific binding mechanism of antibodies to their antigens. The paratope of an antibody is only a tiny fraction of the whole antibody. Therefore, the question arises whether a smaller part of the antibody can be used to create a smaller label with conserved binding affinity. Figure 1-2 shows a more detailed scheme of a conventional and heavy chain antibody. The heavy chain is shown in blue, the light chain in green. It was shown that isolated heavy and light chains of antibodies are still able to bind specifically, although their affinity is often reduced. In 1993, it was discovered that camelids produce functional antibodies which lack the light chain completely (Figure 1-2 b).

![Figure 1-2 Scheme of a conventional and heavy chain antibody. (a) shows a conventional antibody. The heavy chain is shown in blue, the light chain in green. In (b) a heavy chain antibody is shown. It is produced by camelids. At the N-terminal domain of the heavy chain antibody there is the VHH called N-terminal domain that is sufficient for antigen binding. Adapted from Harmsen.](image2)

The single N-terminal domain of the heavy chain antibodies (VHH), also referred to as nanobodies, shows both desired properties: high binding affinity and a small size.
Similar to antibodies, nanobodies can be labeled with fluorophores as well and can be used for IHC. Their advantage compared to other antibodies is the reduced size which leads to a better tissue penetration and the recognition of otherwise hidden antigenic sites\textsuperscript{11}.

### 1.1.2 Phalloidin

Phalloidin is another small label that, tagged with a fluorophore, can be used for IHC. Phalloidin is a toxin that can be found in the death cap mushroom and functions by stabilizing filamentous actin (F-actin) and preventing the depolymerization of actin fibers\textsuperscript{12}. Due to the specific binding to actin filaments phalloidin is widely used in live sciences to investigate actin. Its advantage compared to antibodies against actin monomers is threefold. It is much smaller than conventional antibodies reducing the distance between epitope and fluorescent marker drastically, it specifically targets filamentous actin and it has a higher labeling efficiency than antibodies against actin\textsuperscript{13}.

### 1.1.3 Green fluorescent protein

The Green Fluorescent Protein (GFP) was discovered in the early 1960s by Shimomura et al.\textsuperscript{14} in the jellyfish Aequorea victoria. It was purified and characterized in 1971 by Morise et al.\textsuperscript{15}. GFP has a barrel structure with the chromophore enclosed in the barrel as can be seen in Figure 1-3. With a size of approximately 27 kDa it has a relatively large structure compared to other fluorescent markers. Its emission peak lies at 509 nm. The crucial breakthrough of GFP came with the cloning of its gene\textsuperscript{16} and the demonstration that when expressed in other species than jellyfish it still shows fluorescence\textsuperscript{17}. GFP can either be used as a tag for proteins of interest or as a reporter. Depending on how GFP was introduced to the cell it can be heritable. Using a knock-in method, GFP can be attached to all expressed proteins of a cell without the need of invasive methods. Due to the great potential of GFP, many different mutants have been engineered, improving the excitation wavelength\textsuperscript{18}, folding efficiency\textsuperscript{19} and leading to enhanced GFP (EGFP) or different colors like the yellow fluorescent protein (YFP) or a photoactivatable GFP variant called PA-GFP (photoactivatable GFP)\textsuperscript{20}. Another advantage of GFP is that it is directly attached to the protein of interest since no label is required.

![Figure 1-3 Green Fluorescent Protein. All GFP derivatives show a similar structure which contains the fluorophore in the center of a barrel formed by eleven β-strands (PDB: 1GFL).](image-url)
1.1.4 His-tag and SNAP-tag

Another option of labeling is using small tags like the SNAP\(^{21}\) or His-tag\(^{22}\). In both cases, in the first step, the tag is genetically fused to a protein of interest. In a second step, a fluorophore labeled counterpart is used to attach fluorescent dyes to the tagged target protein. When working with His-tags the fluorophore is coupled to tris-N-nitrilotriacetic acid (Ni-trisNTA) which reversibly binds to the His-tag. In contrast, using SNAP-tags BG-NH\(_2\) is coupled to the fluorophore and binds covalently. In comparison to genetically expressed fluorophores a wider range of fluorophores can be applied.

His-tags provide two benefits compared to SNAP-tags. First, the binding is reversible when applying Ethylenediaminetetraacetic acid (EDTA) or imidazole the Ni-trisNTA can be eluted\(^{23}\), while the SNAP-tag – BG-NH\(_2\) (benzylguanine) binding is covalent and not reversible. Second, the His-tag is significantly smaller and therefore easier to attach to the protein of interest and thus the chance of an altered function of the target protein is reduced.

1.2 Optics

The basic mechanism of modern light microscopy is to collect light emitted by fluorophores and focus it on a detector. This section covers how the light propagates through media, what happens at the borders between different media and why and how these phenomena have an influence on the quality and appearance of images.

First the concept of the wave nature of light is introduced which helps to understand important phenomena of optics like refraction, chromatic aberration and the diffraction limit. The last part of this section will introduce the term of resolution.

1.2.1 Wave nature of light

Many phenomena of light can be explained by thinking of light as an electro-magnetic wave. The wave nature of light was first proposed by Christiaan Huygens in 1678. Augustin-Jean Fresnel showed in 1816 that Huygens theory explains diffraction effects and the rectilinear propagation of light. The Huygens-Fresnel principle states that every point on a wave front can be considered as the origin of a spherical wave (Figure 1-4). In this theory, the assumption is made that the spherical waves only travel in the “forward” direction. Despite this arbitrary assumption without an obvious physical basis, the Huygens-Fresnel principle predicted phenomena like the Argo spot, a bright spot in the center of the shadow cast by a spherical object which cannot be explained by geometrical optics.

![Figure 1-4 Huygens-Fresnel Principle. Each point on a wave front can be considered as the origin of a spherical wave. The superposition of the waves create a new wave front.](image)

The propagation speed of a light wave is dependent on the media it is passing through. The speed of light is approximately \(c_0 = 10^8\) m/s in vacuum but slower in other media. The ratio between the speed of light in vacuum and another medium is called the refraction index.
\[ n = \frac{c_0}{c_1} \]  

(1.1)

c_0 is the speed of light in vacuum, c_1 describes the speed of light in a given medium and n is the refractive index of that medium.

The refractive index also has an influence on the wavelength of light while it is passing a medium, since frequency, wavelength and propagation speed are related as in Equation (1.2).

\[ c = \frac{c_0}{n} = \nu \lambda \]  

(1.2)

c denotes the speed of light in the respective medium, \( \nu \) describes the frequency and \( \lambda \) the wavelength of light in the given medium.

The frequency is constant regardless of the medium, resulting in a decreased wavelength compared to the vacuum.

### 1.2.2 Refraction

Refraction occurs at the border between two media that have different refractive indices. It can be observed if a beam of light passes from one medium to another at an angle which differs from zero degrees. How the path of the light is altered at the surface between two different media is described by Snell’s law as shown in Equation (1.3).

\[ \frac{\sin \theta_1}{\sin \theta_2} = \frac{c_1}{c_2} = \frac{\lambda_2}{\lambda_2} = \frac{n_2}{n_1} \]  

(1.3)

\( \theta \) describes the angles, measured in relation to the surface normal, \( c \) the velocities, \( \lambda \) the wavelengths and \( n \) the refractive indices in both media. Figure 1-5 shows three cases of refraction. Under steep angles, the beam of light will be refracted away from the normal vector of the surface (a). If the angle of incident increases further and further, a critical angle \( \theta_c \) is reached (b). For larger angles the beam will not be able to leave the media but total internal reflection (TIR) will occur (c). The beam of light is reflected under the same angle it hit the surface.

Total internal reflection only occurs if the beam of light propagates within the optically denser medium and hits the surface to the optically less dense medium.

![Figure 1-5 Refraction under different angles. In this illustration, the lower medium is the optically denser medium. For small angles \( \theta \), the beam will be bent away from the surface normal (a). For the critical angle, the refracted beam path is parallel to the surface (b). For any larger angle, total internal reflection occurs (c).](image)

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6
1.2.3 Chromatic aberration

The speed of light in different media is dependent on the refractive index of the medium (Equation (1.1), (1.2) and (1.3)). The refractive index has influence on the way the light is refracted at the border of the medium. The refraction index of each medium is dependent on the wavelength. This results in slightly different beam paths of the light depending on its color.

For fluorescence microscopy this has the following implication. Collecting lenses are made of glass and refract the incoming parallel light in such a way that it is focused in the focal point. Different wavelengths result in different refraction and therefore in a slightly altered focal point. This phenomenon is depicted in Figure 1-6. Chromatic aberration can be corrected for, but will still lead to distortions in the range of several tens of nanometers. This does barely affect image acquisition for diffraction limited microscopy but has a strong influence on the quality of super resolution imaging for which the distortions have the same order of magnitude as the resolution of the technique itself.

![Figure 1-6 Illustration of chromatic aberration on a collecting lens. The focal point of the lens is shifted for beams of light of different colors.](image)

1.2.4 Diffraction limit

The diffraction limit describes a limited resolution achievable in conventional light microscopy. This limit was first described by Ernst Abbe in 1873. It originates from the wave nature of light and cannot be overcome by better components of the microscope like improved lenses.

The condition for the diffraction limit \( d \) Abbe found is given by Equation (1.4):

\[
d < \frac{\lambda_0}{2n \sin \alpha_{\text{max}}}
\]

The angle \( \alpha_{\text{max}} \) describes the maximal half-angle under which light is collected by the objective, \( n \) is the refractive index of the immersion medium and \( \lambda_0 \) the wavelength of the fluorophore that is used.

Using the following identity that defines the numerical aperture (NA) of an optical system,

\[
NA = n \sin \alpha_{\text{max}}
\]

Equation (1.4) reads as follows:

\[
d < \frac{\lambda_0}{2NA}
\]

For a red-emitting fluorophore such as Alexa Fluor 647, and a NA of 1.45 Equation (1.6) predicts a limit of approximately 220 nm. This is small compared to whole cells but still large with respect to protein complexes.
1.2.5 Airy distribution

The diffraction limited intensity pattern can be motivated as follows. When a fluorophore in the focus of the objective lens (Figure 1-7) is imagined, a segment of a spherical wave hits the objective lens and is transformed into a parallel wave front. The Huygens-Fresnel principle states that each point of a parallel wave can be imagined as the source of a Huygens wavelet. If the back focal plane is considered to be the origin of the Huygens wavelets, the projection of these wavelets by the tube lens onto the image plane will show a bright spot in the center. This is where all wavelets interfere constructively because there the optical path length of all wavelets is identical. Further away from the center of the image the interference becomes less and less constructive until there is a region of destructive interference. There are more local intensity maxima with increasing distance from the center. The intensities of these local maxima are decreasing. The intensity distribution is rotationally symmetrical. Within the focal plane the intensity distribution \( I(\nu) \) is described by the Airy distribution Equation (1.7).

\[
I(\nu) = I_0 \left( \frac{2J_1(\nu)}{\nu} \right)^2 \tag{1.7}
\]

Where \( I_0 \) describes the intensity of the Airy distribution at its center, \( J_1 \) is the Bessel function of order one and of the first kind, \( \nu \) is defined by Equation (1.8).

\[
\nu(r) = \frac{2\pi NA r}{\lambda} \tag{1.8}
\]

\( r \) stands for distance to the center, \( NA \) for the numerical aperture and \( \lambda \) for the wavelength.

![Figure 1-7 Illustration of the origin of the point spread function. Wave fronts are depicted as dotted lines (adapted from Fluorescence Microscopy : From Principles to Biological Applications)](image)

The Airy-disc describes the intensity distribution of a diffraction limited point-like object. It can be approximated by a Gaussian distribution with the appropriate width as shown in Figure 1-8.
1.2.6 Resolution

Every imaging system has a limited resolving power. Below a certain scale, depending on the imaging system, small details will not be present in the acquired image. The resolution of an imaging system describes the scale to which details will be captured. For conventional light microscopy, diffraction is the main contribution to a limited resolution at high magnifications. This is due to the blurred appearance and hence overlapping of the signals of individual fluorophores which smooths out details that are below the order of magnitude of the point spread function.

The resolution can be defined by the distance between two fluorophores that can be distinguished. One definition of what distinguishable means was defined by Sir Rayleigh. His definition, called the Rayleigh criterion, states that two objects can be distinguished as long as the maximum of one Airy distribution falls onto the first minimum of the Airy distribution of the second object. This is illustrated in Figure 1-9. For a red emitting fluorophore the calculated diffraction limit (see section 1.2.4) is approximately 200 nm. This diffraction limited resolution is sufficient to investigate large cellular compartments like mitochondria or the nucleus but prevents studies of smaller structures like vesicles or the nuclear pore complex.
1.3 Microscopy Techniques

The human eye has limited resolving power. To examine smaller objects, magnifying devices have been invented. To give a brief overview, in this section different microscopy techniques with their inherent advantages and disadvantages will be described and discussed with respect to the goals of this work.

1.3.1 Conventional light and fluorescence microscopy

The basic principle of a microscope is shown in Figure 1-10. The object of interest, in this example a ladybug, is placed the focal distance apart from the center of the objective lens. Light transmitted through the sample (bright field) or emission light from the sample is collected by the objective lens and focused onto its image plane. Subsequently, this light is collected by the eyepiece. Light beams from one spot in the original image are refracted to the same spot on the retina. If a camera is used instead of the eye, an additional lens is needed that performs the task of the lens of the human eye. In this case, the camera is positioned at the imaging plane of this additional lens.

![Figure 1-10 Scheme of a simple microscope in relation to the perception by the human eye. The small ladybug on the left is magnified by the objective and the eyepiece lens. For the eye it appears to be much larger than its actual size.](image)

1.3.1.1 Widefield fluorescence microscopy

In fluorescent microscopy, monochromatic light is used to illuminate the sample which was stained with fluorescent tags. There are different ways to illuminate the sample as depicted in Figure 1-11. The easiest and most common way to excite the sample is called epi-illumination. In this setup, after the excitation beam is focused on the back focal plane of the objective, it becomes parallel, exits the objective and illuminates a column of the sample. Since the whole column is excited, emission light from fluorophores outside the focal volume is recorded as well, which creates a fluorescent background and reduces the signal-to-noise ratio.

In the case of HILO (highly inclined and laminated optical sheet) illumination the excitation beam is shifted from the central position further outwards. This results in a refracted beam that exits the objective in a certain angle. Subsequently, the beam of light hits the upper glass surface and is further refracted. The resulting light sheets are typically about 4 μm thick and can be adjusted to be almost parallel to the glass surface, thereby allowing their passage through the sample. This illumination excites only a band of 4 μm thickness and no fluorophores above or below. This leads to less background fluorescence from out of focus regions in thick samples.

If the shift of the excitation beam is further increased, the light beam is not refracted at the glass surface but is totally internally reflected (TIR). Despite the total reflection, an evanescent electromagnetic field is induced at the surface of the glass. Its intensity is decreasing exponentially with increasing depth, but it is still strong enough to excite fluorophores within the
first 100 to 200 nm of the sample. Therefore, the fluorescence from other planes is greatly decreased but only the signal of a small fraction of the sample directly on the glass surface can be imaged.

Another option is to illuminate the sample not via the objective which is used for detection but with an orthogonal light source. This idea was first published in 1903 by Siedentopf and Zsigmondy\textsuperscript{31} using sunlight for illumination. In 1993, light sheet microscopy was redeveloped\textsuperscript{32}. Usually, a laser beam is focused by a cylindrical lens, a lens which only focuses light along one axis. Light sheet illumination reduces background from out of focus regions and at the same time illuminates the focal volume completely. A disadvantage arising from this broad illumination is that the sample is photobleached also beyond the field of view.

Figure 1-11 Illustration of different illumination modes. In epi-illumination the excitation light is focused at the back focal plane of the objective and leaves the objective in a parallel mode. For HILO illumination (highly inclined and laminated optical sheet) the excitation beam is displaced from the central axis of the objective, as a result a thin light sheet which leaves the objective under a large angle close to 90 degree is created. Even more displaced excitation beams are used for TIRF (total internal reflection and an evanescent field) illumination, leading to TIR and an evanescent field that excites the first 100 to 200 nm of the sample. A laser beam, focused by a cylindrical lens, positioned orthogonally to the axis of the objective is used for light sheet illumination, exciting only a small band of fluorophores in the focal volume of the sample. (Figure augmented from Flottmann\textsuperscript{29}).

In light microscopy the magnification can be freely adjusted by replacing the lenses, thus large volumes can be imaged quickly. The reasonable magnification is only limited by diffraction yielding lateral resolutions of about 200 nm and axial resolutions of about 500 nm. Due to the limited resolution light microscopy is the wrong tool to study the spatial arrangement of proteins, which requires a higher resolving power.

1.3.1.2 Confocal microscopy

In confocal microscopy, a pinhole is introduced to the emission beam path. The limited aperture of the pinhole reduces the amount of light that reaches the camera from light sources further away from the focal plane. This leads to higher contrast and to an improved resolution along the z-axis.

In contrast to widefield fluorescent microscopy, for confocal microscopy predominantly a point-scanning illumination technique is used. Only a small column of the field of view is illuminated at a time. The emission light from this excited volume is then detected by a photomultiplier tube. The final image is formed by assigning the detected intensity to each scanned position.
Introduction

In case of a scanning confocal microscope, the capturing of an image takes more time compared to a conventional microscope. Artifacts can be created by fast movements within the sample. Since the image is scanned pixel by pixel and line by line, an object traveling along the axis of image acquisition will for example appear elongated. Confocal microscopy can be applied to the same samples as light microscopy, but shares its limitations in resolution. The biggest advantage of confocal microscopy (without applied deconvolution) compared to conventional light microscopy is a higher signal to noise ratio which results in a higher image quality.

1.3.2 Super-resolution microscopy

There are many questions that can be answered already with confocal or conventional light microscopy. However, the resolution for these techniques is limited to approximately 200 nm and thus certain questions, like the number of vesicles at the active zone of a synapse, remain unanswerable. In the last decade, new techniques with resolutions way beyond the diffraction limit were developed. These super-resolution techniques fall into two categories.

One category is the targeted approach, where the sample is illuminated with certain light patterns that limit the detection of fluorophores to regions smaller than in conventional light microscopy. Stimulated emission depletion (STED) makes use of a reduced effective size of the excitation spot to gain lateral resolutions in the range of 30 nm and axial resolutions of about 50 nm. Another example of the targeted approach is saturated structure illumination microscopy (SSIM) where periodic line patterns are used for illumination and resolutions of 100 nm lateral and 300 nm axial are achieved.

The second category is called localization-based super-resolution microscopy. It was invented independently by three research groups in 2006. It comprises photoactivated localization microscopy (PALM), fluorescence photoactivation localization microscopy and stochastic optical reconstruction microscopy (STORM). The principle behind these localization based super-resolution methods is that only a subpopulation of all fluorophores of a sample is emitting light at the same time and thus the position of each diffraction limited spot can be detected with higher accuracy. To obtain the final image, the positions of individual fluorophores recorded in thousands of frames have to be projected onto one single image. There will be a more extensive description of single molecule localization microscopy in section 1.3.4. STORM microscopy using a dual objective microscope achieves resolutions of 10 nm in lateral and 20 nm in axial dimension.
Taken together, SRM can reach resolutions in the range of 10-50 nm laterally and 20-60 nm axially. The most recent member of SRM is MINIFLUX, a technique expanding optical microscopy to a resolution of 1 nm. Hence, these techniques can be used to study the molecular architecture of cellular machines. Compared to widefield and confocal microscopy SRM provides approximately a tenfold increase in resolution along each axis.

1.3.3 Electron microscopy
The first electron microscope was built in 1931 by Knoll and Ruska. Instead of visible light, highly accelerated electrons are used to probe the sample. With modern electron microscopes subnanometer resolutions are achievable. In transmission electron microscopy (TEM) a beam of electrons is focused on the sample by electromagnetic lenses and an image is created by detecting differences in the number of scattered electrons that depend on the characteristics of the material penetrated by the beam. In scanning electron microscopy, the focused electron beam is translated across the specimen. Due to the interaction of the beam with the specimen, multiple effects like backscattering of electrons or the creation of secondary electrons take place. These signals carry information about the surface of the object that was hit by the electron beam and can be translated to intensity values of that specific location. The final image is created by assigning the detected intensities to the scanned positions, similar to the image formation process in scanning confocal microscopy. The resolution depends on the size of the electron beam which is in the order of nanometers.

To create 3D images using EM, electron tomography can be used. For this method the 3D image is calculated from multiple 2D images recorded under different angles. Resolutions of about 2 nm are achievable.

Electron microscopy provides the best resolving power achievable for biological samples but also has certain limitations. More complex sample preparation is necessary. For transmission EM, the sample has to be thin enough for the electron beam to penetrate. For scanning EM, the sample has to be conductive to prevent charge build-up. The major disadvantage of EM is that it is not possible to get 3D information of molecular identities. In summary, EM provides both lateral and axial molecular resolutions, but lacks labels, thus making it hard to distinguish the molecular identity of filaments of similar size, for example.

1.3.4 Single molecule localization microscopy
Single molecule localization microscopy (SMLM) is one kind of super-resolution technique as briefly mentioned in section 1.3.2. It has been chosen as the major imaging method for this thesis because of the very high resolution, multi-color imaging and 3D imaging capabilities. Also, this can be achieved using a regular light microscope without complicated modifications (in comparison to STED). In this combination it reflects the currently best approach to decipher the molecular architecture of cells and tissues. This section will give an introduction to the most important aspects of SMLM.

1.3.4.1 Theoretical localization precision
Single molecule localization microscopy eludes the diffraction limit by finding the center of individual point spread functions (PSFs) with high precision. Therefore, the Rayleigh criterion is not a valid measure for the resolution in SMLM. The position of a single fluorophore can be determined with high accuracy as described by Thompson et al. The localization uncertainty or localization precision $\Delta x$, describes the spread of the estimated positions around the true position of the light source. It decreases the more photons are captured by the detector, typically a
camera with a high quantum efficiency. Given the number of collected photons $N$ and the standard deviation $\sigma$ of the PSF, the localization error $\Delta x$ can be approximated by Equation (1.9).

$$\langle (\Delta x)^2 \rangle \approx \frac{\sigma^2}{N}$$  \hspace{1cm} (1.9)

This approximation ignores deviations originating from background noise and the influence of the pixilation, noise that originates from the uncertainty where on the camera pixel the photons arrived.

If these effects are also taken into consideration, the localization error is described by Equation (1.10).

$$\langle (\Delta x)^2 \rangle = \frac{\sigma^2 + a^2}{12 N} + \frac{8 \pi \sigma^4 b^2}{a^2 N^2}$$  \hspace{1cm} (1.10)

Where $a$ stands for the pixel size and $b$ describes additional background.

A similar formula estimating the localization precision was published by Mortensen$^{54}$. The localization precision is given by Equation (1.11).

$$\sigma_a = \sigma + \frac{a^2}{12}$$  \hspace{1cm} (1.11)

$\sigma_a = \sigma + \frac{a^2}{12}$ describes the standard deviation of the PSF but is corrected for pixilation noise.

There is a published method to estimate the localization precision directly from the acquired image stack$^{55}$. In this approach, the distance between positions occurring in consecutive frames, most likely originating from one blinking event, is used to estimate the underlying localization precision. The localization precision can also be estimated from single fluorophores that emit light multiple times. The standard deviation of the positions for each fluorophore gives a good estimate of the localization precision$^{46}$. Another approach employs the Fourier ring correlation between two subsets of all localizations to estimate the smallest features preserved in the SMLM image$^{56}$. The localization precision translates to resolution by multiplying it with a factor of 2.3$^{57}$.

1.3.4.2 Photoswitching fluorophores

The key principle of SMLM is the detection of many sparsely distributed PSFs of blinking fluorophores. Only certain fluorophores can be used for SMLM experiments. A fluorophore that switches from a fluorescent state to a non-detectable state is called a photoswitching fluorophore. There are fluorophores that switch only once from the dark state to the fluorescent state and back to a final dark state. Reversibly switching fluorophores can perform many transitions between the dark and light-emitting state. Eventually, the fluorophore will irreversibly photobleach and stay dark. Organic dyes are the group of fluorophores mostly used for direct STORM (dSTORM)$^{58}$ measurements. They exhibit bright fluorescence and an extended π-electron system. Prominent groups of organic dyes are carbocyanines, oxazines and rhodamines. The whole visible wavelength spectrum can be covered with these dyes$^{59,60}$. Potential mechanism behind the photoswitching of carbocyanines$^{61}$, oxazines$^{62}$ and rhodamines$^{63}$ have been reported.

For all of these dyes there is the necessity of a switching buffer, which provides thiols like β-mercaptoethanol (β-ME) or β-mercaptopethylamine (MEA) in a phosphate buffer (PBS)$^{60,64}$. For Carbocyanines it has been shown that they form an encounter complex with the thiol anion when excited with red light. While the complex is formed the dye cannot emit light. The formation of
this complex has been shown with mass spectrometry\textsuperscript{61}. Irradiation with UV light decomposes the complex and recovers the carbocyanine.

A simple model of the photoswitching kinetics is shown in Figure 1-13. \( k_a \) describes the transition rate from the dark to the fluorescent state, \( k_d \) the transition rate from the fluorescent to the dark state and \( k_b \) the transition rate from fluorescent to the photobleached state. Other possible dark states or not depicted.

During the active state a fluorophore emits up to several thousands of photons. A part of these photons is collected by the objective and eventually forms a diffraction-limited image of the fluorophore on the camera. The photons follow the distribution given by the PSF. All photons captured from the emission of a single fluorophore during its active state are also called a blinking event.

### Figure 1-13 Simple model of the photoswitching kinetic. D denotes the dark state, B the photobleached state and A the fluorescent state. Arrows indicate transitions, the transition rates are written above the arrows.

Successful SMLM experiments rely on the temporal separation of densely packed fluorophores. Therefore, the vast majority of all fluorophores have to be in the dark state and only a small fraction should occur in the active state. The number of fluorophores in the dark and active state is determined by the total number of fluorophores and by the transition rate constants \( k_a \) and \( k_d \). The ratio \( r \) of both numbers (Equation (3.1)) is therefore crucial.

\[
r = \frac{k_a}{k_d}
\]  

(1.12)

If \( r \) is too large, the number of fluorophores per frame is too high and the individual PSFs have a high chance to overlap, if \( r \) is too low the measurement takes longer than necessary.

During a measurement the number of bleached fluorophores increases. This results in a decreasing number of fluorophores in both the active and the dark state. To keep the absolute number of fluorophores in the active state constant, the transition rate from the dark to the active state needs to be increased. This can be realized by continuously increasing additional UV illumination of the sample.

#### 1.3.4.3 Astigmatism based 3D SMLM

A new approach to acquire not only the \( x \) and \( y \) position of the PSF but also information about the \( z \) position was first published for single particle tracking in 1994\textsuperscript{65}. In 2008, Huang et al. used this approach to perform the first 3D-STMOR measurement on microtubules and clathrin-coated pits\textsuperscript{66}. For astigmatism based 3D-STMOR a cylindrical lens with a large focal length is placed into the detection pathway of an epi-fluorescence microscope. The cylindrical lens breaks the symmetry of the PSF along the \( z \)-axis.

The principle of the astigmatism approach is shown in Figure 1-14. The cylindrical lens focuses the beam of light only along one dimension. The result is that the PSF cannot be focused perfectly
along both axes any more. Either it is perfectly focused along the $x$-axis but then the PSF is wider along the $y$-axis or vice versa. This results in elliptical instead of spherical PSFs. Between the two focus positions the PSF is a superposition of both ellipses and appears to be almost spherical. To assign $z$-values to individual blinking events, a calibration curve is recorded. To this end, a PSF is recorded over a large range of $z$-positions and the width in both spatial dimensions is determined. This relation between the known $z$-position and the shape of the PSF can be used to assign $z$-values for PSFs of unknown $z$-position by comparison.

Figure 1-14 Principle of astigmatism-based 3D-STORM. In (a), a schematic drawing of the placement of the cylindrical lens between the objective and the tube lens is shown. The PSFs shape is altered depending on the focal position of the fluorophore (b). In (c), a calibration curve is shown where the shape of the PSF was measured at known focal positions. The black dots correspond to the width of the PSF in $x$-, the white dots in $y$-direction. Figure adapted from Huang.

In addition to the astigmatism approach, several other, yet more complicated, methods have been developed for 3D imaging such as biplane detection, double-helix and interferometric PALM. For biplane detection an additional beam splitter is necessary which creates two beam paths of slightly different length. By comparison of the two images the axial position of a particle can be localized. The resolution in lateral and axial dimension is better compared to the astigmatism approach but worse than the resolution achieved by the double-helix approach. The disadvantage of this technique is the need of an additional beam splitter in the emission beam path which would reduce the intensities for each channel. If the biplane approach is combined with spectral-demixing, there is no disadvantage compared to the astigmatism approach. For the double-helix approach the emission light is phase modulated and splits the excitation light in two points. The axial information is encoded in the relative position of these points. The localization error stays almost constant over a wide axial range, especially for the axial localization estimation. The downside is a more complicated setup including a spatial light modulator and more complicated image analysis compared to the astigmatism approach. The interferometric approach is the most complex one, it requires two objective multiple detectors and beam splitters but yields excellent axial resolutions.

In summary, there are other approaches that provide slightly better localization precisions in both axial and lateral dimension but also increase the complexity of the setup. The astigmatism approach is induced by inserting a lens into the emission beam path. This lens can easily be removed to achieve better lateral resolutions if no 3D information is needed.
1.3.4.4 Spectral demixing

STORM measurements provide highly resolved images of biological structures. But many questions can only be answered if the position of one protein is known in relation to another protein. To acquire STORM images of two different proteins, two spectrally distinct photoswitchable markers can be used. However due to chromatic aberration, distortions between both images occur. Furthermore, both fluorophores have to be imaged sequentially which might induce photobleaching of the secondly imaged fluorophore during the imaging of the first fluorophore and takes twice the amount of time.

To circumvent the above mentioned problems, in 2012, Lampe et al. published a method based on spectral demixing. For their approach, the authors used two fluorophores with similar chemical properties and overlapping emission spectra, see Figure 1-15. A dichroic-based emission splitter was used to separate the two channels. Due to their similar emission spectra, the emission light of both fluorophores is visible in both channels, but shows different (fluorophore-specific) intensity ratios. One of the two channels is used to detect the position of the fluorophore; the color is assigned according to the intensity ratio of the respective blinking events in both channels.

The advantages are dual color STORM images, free of chromatic aberration and an additional robustness against incorrectly found localizations.

![Closely overlapping emission spectra. The black line indicates the percentage of all photons that are transmitted by the dichroic filter. Adapted from Lampe et al.](image)

**Figure 1-15** Closely overlapping emission spectra. The black line indicates the percentage of all photons that are transmitted by the dichroic filter. Adapted from Lampe et al.¹

1.3.4.5 State of the art of simulation software for SMLM

There are some software packages available that perform simulations of SMLM data. The most flexible software package, TestSTORM provides options to simulate simple predefined 2D and 3D structures with a realistic model for the fluorophores including bleaching. Also, a realistic camera model is used to produce raw Tiff (tagged image file format) stack output using 2D PSF models. Both RapidSTORM and ThunderSTORM provide the option to simulate randomly distributed fluorophores using a realistic model of the fluorophore properties, except bleaching. Also realistic raw Tiff stacks can be simulated using both 2D and 3D PSF models. Additionally, a rendered image of the simulation and a list containing detailed information about the simulated localizations as well as a file containing the ground truth positions, the positions of the simulated fluorophores, is created.
The features missing so far are arbitrary structures in 3D and a more detailed model of the labels including label length and binding angle. However, considering these factors is crucial to get a reliable simulation since for example the label epitope distance introduced by primary and secondary antibody labeling have the same order of magnitude as the achievable resolution of SMLM. Also might the spatial arrangement of the structure have large influence on imaging artifacts as overlapping PSFs.

1.3.4.6 Known challenges in SMLM

Thousands of frames have to be recorded to form an image in SMLM. Thus the image acquisition takes several minutes. Drift of the sample relatively to the objective causes the recorded images to move over time and result in a blurred reconstructed image both in lateral and axial direction. This drift can cause inaccuracies which greatly exceed the resolution of SMLM measurements and have to be corrected, either actively or by post-processing steps. Another challenge for multi-color imaging in SMLM is chromatic aberration which occurs if spectrally distinct fluorophores are recorded. Post-processing or spectral demixing can be used to compensate or circumvent distortions. The probability that two fluorophores emit light within the same frame and in close spatial proximity is higher for densely labeled areas. Overlapping PSFs can lead to an erroneously estimated single location somewhere between both PSFs\(^7\). The length of the label increases the distance between fluorophore and target structure which leads to inaccuracies. Reversibly blinking fluorophores will bleach at a random time point, resulting in missing localizations and a varying number of blinking events per individual fluorophore, which is challenging for quantitative analysis.

1.4 Biological test-systems

To validate a new staining, a new fixation protocol or a new microscope, test samples are needed. Ideally, the molecular composition and the arrangement of the proteins of these test samples are known. This section introduces microtubules and the nuclear pore complex and describes why they were chosen as references.

1.4.1 Microtubules

For the evaluation of the performance of a new microscopy technique, especially in super-resolution microscopy, microtubules are used. Microtubules are part of the cytoskeleton of eukaryotic cells. Besides the maintenance of the cell structure, microtubules play an important role in the intracellular transport.

Microtubules form filaments with a diameter of approximately 25 nm\(^78\). They are hollow and form long cylinders, which are composed of tubulin dimers formed by α- and β-tubulin\(^79\). Thirteen of these dimers form the wall of the microtubules\(^80\). Due to the known structure the exact number of proteins and also their spatial organization is known. Microtubules are often used because they have a large number of binding sites which will lead to images of continuous filaments even for low labeling efficiencies. Another advantage of microtubules is that they are also present at the flat edges of cells where the imaging conditions are ideal.

1.4.2 Nuclear pore complex

The nuclear pore is a big complex formed by a large number of proteins of well-known composition\(^81\). It is located in the membrane of the nucleus. The nuclear pore complex forms a ring made up of 8 unit cells\(^82\) with an outer diameter of approximately 125 nanometers\(^83\).
For experiments the nuclear pore protein, nucleoporin 133, was chosen as a target since it appears in an outer region of the ring and forms an octagon with an edge length of approximately 40 nm. There are four copies of the protein per unit cell and in total 32 copies per nuclear pore\(^8\). The 40 nm distance makes the nuclear pore complex an ideal test system for STORM microscopy with a resolution of around 10 nm. The four-fold multiplicity per unit cell is also beneficial and compensates for low labeling efficiencies.

1.4.3 Mitochondria
Mitochondria can be found in all eukaryotic organisms. They supply energy to the cells and play a role in apoptosis. They differ in shape and size\(^8\) and show a smooth outer membrane. The width of mitochondrial tubes is typically between 250 and 500 nm\(^8\). This z-range can be captured by 3D STORM measurements. Stainings against clusters of outer membrane proteins like TOM20, which are evenly distributed on the surface, appear to be enriched at the rim of the mitochondrion in the projection image and form hollow structures when visualized in 3D. Because of their round shape, Mitochondria are a good test structures for both lateral and axial resolving power.

1.5 Molecular architecture of the active zone in presynaptic nerve terminals
The human brain contains about 100 billion neurons with on average 1000 connections each\(^8\). Signals propagate between neurons through synapses. There are two kinds of synapses. On the one hand, there are electrical synapses, where the connection between two neurons is realized by gap junctions, small conductive connections\(^8\). On the other hand, there are chemical synapses where the conductivity is indirectly realized by the release and the sensation of neurotransmitters.

Chemical synapses consist of two parts, the presynapse and the post-synapse that are separated by the synaptic cleft of approximately 20 nm. An arriving action potential on the presynapse triggers the release of synaptic vesicles (SV), round lipid membrane compartments of approximately 40 nm diameter which contain the neurotransmitters. These neurotransmitters are recognized by postsynaptic receptors generate an electrical signal that propagates further.

![Figure 1-16 Schematic representation of signal propagation through a chemical synapse. (Figure from Lisman\(^8\))](image)

The region of the presynapse where part of the vesicles are stored and released is called the active zone (AZ). A crucial property of synaptic transmission is the release probability. Not every action potential arriving in the presynapse triggers the fusion of a synaptic vesicle\(^\text{90}\). The underlying processes are not fully understood yet.
In this thesis the calyx of Held, an excitatory giant synapse of the mammalian auditory brainstem was used to investigate the acting and synaptic vesicle distribution. It was chosen since a single calyx has hundreds of AZs and is a well established model system of glutamatergic synapses accessible to direct molecular perturbation.

1.5.1 Synaptic vesicles
There are two different modes of SV release, either spontaneous release or release triggered by an arriving action potential. The basic processes that lead to the triggered release of a vesicle are the depolarization of the presynaptic cell by the opening of sodium channels, which leads to the opening of voltage-gated calcium channels that mediate several processes leading to vesicle fusion with the plasma membrane and to release of neurotransmitters into the synaptic cleft.

Three different groups or pools of vesicles are proposed with different functions each. The ready-releasable pool contains vesicles that are directly docked to the plasma membrane and can be fused directly on stimulation. The recycling pool contains vesicles that are in close proximity to the plasma membrane and is replenished upon moderate stimulation. Vesicles of the third pool, the reserve pool, are only released on strong stimulation and account for the majority of all vesicles. While synaptic vesicles are well characterized functionally, the molecular correlate of the different SV pools remains unknown. 3D SMLM should prove the resolution to decipher the molecular composition of the functionally defined SV pools. There are publications that suggest that there are SV which at least in part show distinct molecular compositions and show also different release behavior. The average protein composition of SVs is also known and led to the model of a SV as shown in Figure 1-17. Synaptophysin is the second most abundant protein in the membrane of SVs and therefore a good SV marker.

![Molecular model of an average SV. Figure from Takamori et al.](image-url)
1.5.2 Actin

Actin is part of the cytoskeleton of eukaryotic cells. In its filamentous form, which is built by the polymerization of monomeric G-actin, it is called F-actin. Actin filaments can be assembled and disassembled very dynamically. The function of the actin filaments can, for example, be cell movement\(^{96}\) or stabilization as at the membrane of erythrocytes\(^{97}\) where it forms filaments of 14 – 16 monomers\(^ {98}\). Actin interacts with many other proteins of the cytomatrix of the active zone\(^ {99,100}\) and might also be involved in the clustering and recruitment of vesicles\(^ {101,102}\). Hirokawa et al.\(^ {103}\) proposed a model of the presynaptic terminals based on quick-freeze deep-etch electron microscopy images (see Figure 1-17). In this model actin forms filaments that proceed from the membrane of the active zone inwards and might be used for active SV transport. The model also shows that SVs are linked by short filaments to each other and the cytoskeleton within the presynapse. The identity of the small linkers attached to the vesicles remains unknown\(^ {104}\). Synapsin, with its ability to bind to actin and reversibly to SVs might be responsible for linking the vesicles to the actin scaffold\(^ {105,106}\), making actin a possible candidate for the linker. Other publications indicate that actin can be found around SV clusters in high abundance but little actin is found within the SV cluster itself\(^ {101}\).

\[\text{Figure 1-18 Model of the presynaptic terminals based on the study of Hirokawa et al.}\]

Actin filaments (a) and microtubules (mt) are depicted as the main cytoskeletal elements. Synaptic vesicles (SV) are linked to each other and the cytoskeleton by short strands. Figure from Hirokawa et al.\(^ {103}\).
1.6 Aim of the study

This work addresses a technical and a biological problem. The technical problem is to quantitatively understand the limits of STORM microscopy in resolving the nanostructure of biological systems. The biological problem aims at understanding the nanoarchitecture of presynaptic vesicle clusters in synapses of the mammalian central nervous system. Both aspects are tightly intertwined, the former will define the interpretations of the nanostructural representations of the latter.

1.6.1 Technical aims

Since the active zone is a small and densely packed region conventional light microscopy does not yield a sufficient resolution to resolve its architecture. Therefore a 3D dual color dSTORM microscope using spectral demixing needs to be designed and built. Together with a newly developed post-processing software an imaging workflow will be established which will give the opportunity to investigate the arrangement of two proteins of the active zone with respect to each other.

To interpret the results of the measurements and investigate the limits of SMLM a simulation tool needs to be developed, which helps to simulate the expected outcome of a SMLM experiment and gives the opportunity to test hypothesis.

1.6.2 Biological aims

To investigate the nanoarchitecture of presynaptic vesicle clusters, two proteins of interest, actin and synaptophysin, are the focus of this work. Synaptophysin is a protein with high abundance in SVs and therefore a good marker for SVs. To answer the question about the role of F-actin within the presynapse it will be labeled with phalloidin, a very small label which binds specifically to F-actin. Dual color 3D dSTORM measurements of the calyx of Held will be performed using the new microscope and analyzed. We seek to answer the question about the F-actin distribution in the SV cluster. The expectations are to find long actin strands within the presynapse, a fine network connecting SVs or an actin cage around the SV cluster.
2 Materials and methods

This section summarizes the preparation steps for the samples and describes the imaging conditions for the measurements shown in this thesis. The microscope that was built will be introduced followed by the description of the parameters used for the simulations shown in the Results part.

2.1 Sample preparation and labeling

2.1.1 Synaptophysin 1 and F-actin at the calyx of Held

All animal experiments were conducted in accordance with the German animal welfare guidelines and were approved by the responsible authority (Regierungspräsidium Karlsruhe).

A 9-day-old Sprague Dawley rat was deeply anesthetized using isoflurane. After that it was chemically fixed by transcardial perfusion with 20 ml PBS. Followed by 40 ml of 4% PFA and 0.5% glutaraldehyde (GA) (SERVA Electrophoresis, Heidelberg) solved in PBS. The rats' brain was removed and post-fixed at 4 °C over night in the fixative described above. The brain was sliced in sections of 200 μm thickness using a vibratome (Sigmann Elektronik, Hüffenhardt). The medial nucleus of the trapezoid body was excised manually using a razor blade.

Small cuboid parts of the size of approximately 500 μm times 300 μm times 200 μm were prepared. The prepared blocks were incubated in 2.1 M sucrose in 0.1 M PBS (pH 7.4) for 30 min. The tissue block was mounted on an aluminum holder and frozen in liquid nitrogen. The frozen block was put into a cryo-ultramicrotome (Leica Ultracut S equipped with a cryo chamber; Leica Microsystems, Vienna). The chamber temperature was set to -80 °C, the specimen temperature to -80 °C and the knife temperature to -70 °C.

Cryosections of 800 nm thickness were cut and picked up with a perfect loop (Diatome, Switzerland) in 2.3 M sucrose in PBS and put on glass-bottom dishes (coverslip thickness grade 0). The cryosections were thawed for 10 min and washed three times for 5 min with PBS. The sections were blocked with 0.5% FCS in PBS for 30 min and incubated with primary antibodies against synaptophysin 1 (Synaptic Systems, 101011) at a concentration of 1:500 in blocking solution for 1 h. After washing the sample three times using blocking solution, the sections were incubated with CF680 labeled secondary antibodies against mouse (Sigma, SAB4600199) using a concentration of 1:500, and phalloidin coupled to Alexa Fluor 647 (Life Technologies) using a concentration of 1:13, for 1 h. After three washing steps, the sample was post-fixated for 10 min using 4% PFA.

2.1.2 Microtubules

COS-7 cells (Sigma-Aldrich, 87021302-1VL) were fixed using methanol at -20 °C for 5 min, washed and stored overnight in PBS (phosphate buffered saline). For all washing steps the PBS was removed after 5 min. The next day the cells were blocked using 5% horse serum in PBS. The primary antibody against α-tubulin (Sigma, T6199) was applied in a concentration of 1:500 in PBS and incubated for 1 h. Cells were washed three times with blocking buffer (5% horse serum in PBS). Secondary antibodies from mouse immunoglobulin-γ (IgG) (Invitrogen, A-21236) coupled to Alexa Fluor 647 were used in a concentration of 1:500 in PBS and incubated for 1 h. Afterward, the cells were washed three times with PBS and postfixed with 4% paraformaldehyde (PFA) for 10 min.
2.1.3 Mitochondria

COS-7 cells (Sigma-Aldrich, 87021302-1VL) were fixed using 4% PFA at room temperature for 30 min. After washing three times with PBS, Glycin (50 mM in PBS) was applied for 10 min. The cells were blocked with 5% FCS (fetal calf serum) in PBS for 30 min. Subsequently, the primary antibody against TOM20 (Santa Cruz Biotechnology, sc-11415) with a concentration of 1:500 in blocking solution was incubated for 1h. After three washing steps the secondary antibodies against rabbit (Sigma, SAB4600362-125 UL), with CF680 conjugated to the antibody, were applied in a concentration of 1:500 in blocking solution. After washing 3 times, 4% PFA was applied for 10 min and finally removed by three additional washing steps.

2.1.4 F-actin in erythrocytes

Human erythrocytes were fixed using 4% PFA and 0.5% GA (SERVA Electrophoresis, Heidelberg) dissolved in PBS for 30 min. The erythrocytes were washed three times and gently centrifuged. 150 μl of the erythrocyte solution was put on glass-bottom dishes that were coated with poly-L-lysine and concanavalin A. For blocking 5% FCS in PBS was applied for 30 min. After that the sample was incubated with 6.6 μM phalloidin, coupled to Alexa Fluor 647 (Life Technologies) in blocking solution for 1 h.

2.1.5 SNAPf-tagged nup133

For the preparation of nup133 the protocol described here was used. Summarized, a stable, SNAPf-tagged nup133 expressing U2OS cell line (CLS Cell Line Services, Eppelheim, Germany) was labeled with Alexa Fluor 647.

2.2 Image acquisition and rendering

3D dSTORM imaging was performed on a custom-built microscope described in more detail in the next section. Briefly a 661 nm (Coherent Cube, Coherent, USA) laser was used for excitation. The laser was focused on the back focal plane of a 100× objective (Olympus, Japan) with a numerical aperture of 1.49. Immersion oil with a refraction index of 1.518 (Type F Immersion liquid, Leica, Germany) was used. The emission light passed a tube lens (focal length 18 cm) and was filtered by a multi-bandpass filter (ZT405/488/561/647rpc, Chroma, Bellows Falls, VT). Subsequently the emission light passed a lens (focal length 25 cm) followed by a 700/75 ET bandpass filter (Chroma, BellowFalls, VT) and a 750 nm short-pass filter (Thorlabs). The emission light was split by a custom-made beam splitter (690 nm, Chroma, Bellow Falls, VT). Finally both beams were focused by two lenses with a focal length of 30 cm, on the EMCCD camera (iXon Ultra 897, Andor, UK), which was air cooled to -75 °C. Images were acquired using MicroManager and the continuous imaging mode of the camera.

For active z-focus stabilization a piezo-based objective positioner (Physik-Instrumente, Karlsruhe, Germany) was used. Feedback of an IR beam totally reflected by the cover slip was used and processed by a quadrant photodiode. Its output was used to continuously correct the z-position of the objective.

A conventional fluorescent image was recorded before starting the STORM measurement using a weak laser intensity (~1 W/cm²). To induce photoswitching of the fluorophores the laser intensity was increased (~2 kW/cm²). Additionally to the excitation laser, a 405 nm UV laser with intensity settings continuously increasing during the measurement was used to increase the transition rate from the dark state to the emitting state and to keep the number of blinking events per frame constant. 40000 frames were recorded with an exposure time of 30 ms. For the experiments
described in section 3.3.6.2 varying exposure times of 4 ms, 8 ms, and 16 ms were used and only 5000 frames were recorded. The imaging was repeated resulting in 10000 frames per exposure time in total. For all measurements, 100 mM freshly prepared MEA was used as imaging buffer. RapidSTORM\textsuperscript{75} and the FIJI\textsuperscript{108} plugin ThunderSTORM\textsuperscript{76} were used for image reconstruction. Drift correction, connecting localizations in consecutive frames, demixing and rendering were performed using the post-processing software described in the Results section. For the reconstruction of the single color microtubules sample used for the validation of the post-processing software (see section 3.2.5), a minimal photon number of 1000 photons was used. All STORM images of experimental and simulated data were rendered using the output modules of the post-processing software. A pixel size of 10 nm was used unless stated otherwise. Each localization was rendered using a Gaussian PSF with a standard deviation of 10 nm. The integral of each PSF was equal to the intensity of the localization.

2.3 Programming languages used for this thesis
To create models for SuReSim Matlab\textsuperscript{109} (MathWorks) was used. The control software for the microscope, the post-processing software and SuReSim were all written in Java (Sun Microsystems). For the post-processing software and SuReSim Java 8 or later version are needed, for the microscope control software Java 6 must be used.

2.4 Setup
I built a custom microscope based on the published concepts of demixing\textsuperscript{1} and active focus stabilization\textsuperscript{110}. Dr. Jonas Ries designed the XY-stage and the characteristic of the dichroic mirror used for demixing. I did the selection of the other components, the design of the microscopy stage and the assembly of the microscope.

2.4.1 Overview
Figure 2-1 shows an overview of the components of the microscope. In the upper part the active focus stabilization system (1-3) is shown. The objective (4) is mounted on a piezo-driven stage (5). The dichroic filters (6) and (7) couple the light for the focus stabilization and the excitation beam path (19-27) into the objective (4) and let the light of the emission beam path (8-18) pass. The individual components will be explained in more detail in the following sections.
Figure 2-1 Draft of the custom-built microscope. In the upper part, the active focus stabilization system is shown, consisting of an infrared diode (1) a quadrant diode (2) and a movable mirror for focusing (3). The objective (4) is mounted on a piezo-driven stage (5). The infrared light is coupled into the objective by a dichroic mirror (6). The excitation light is reflected onto the sample using a second dichroic mirror (7). The emission beam path consists of a notch filter (8) that blocks the 661 nm excitation laser (21), followed by the tube lens (9) and a cylindrical lens (10) that can be used to introduce astigmatism to the PSF. At the focal point of the tube lens a slit (11) is placed, followed by another lens (12) that creates a parallel beam again. Different emission filters to block excitation light are placed in a filter wheel (13). The dichroic (14) splits the emission light into two channels that are focused by the two lenses (15 and 16) on the camera (18). One of the two beams is reflected at the inner edge of a prism (17). The excitation light is widened by two lenses forming a telescope (19). The 661 nm laser beam (21) is merged with all the other lasers at the last dichroic (20) of the excitation path. A clean up filter (22) is used for the 661 nm laser (21) to reflect all other wavelengths and transmit only the primary wavelength. The 561 nm laser (23) is merged with the UV-laser (26) and the 488 nm laser (27) using two dichroic mirrors (24 and 25).
2.4.2 Components

Four lasers were used to excite the sample. Two diode lasers (Cube, Coherent, USA) with a wavelength of 661 nm (Figure 2-1; 21) and 405 nm (Figure 2-1; 26) were used as well as two diode lasers (Obis, Coherent, USA) with a wavelength of 488 nm (Figure 2-1; 27) and 561 nm (Figure 2-1; 23). For the 661 nm laser, a clean-up filter (Chroma, USA, ZET660/20x), (Figure 2-1; 22) was used to block all wavelengths except a small band around 660 nm. The beam of the 405 nm laser was merged with the beam of the 488 nm laser using a long pass beam splitter (Chroma, USA, ZT442rdc-UF1) (Figure 2-1; 25). To overlay this combined beam with the 561 nm beam, another long pass beam splitter (Chroma, USA, 525DCXRUUV), (Figure 2-1; 24) was used. Finally, all four beams were coupled together using the beam splitter depicted in Figure 2-1 (20) (Chroma, USA, 590dxcx). Two coated lenses with a focal length of one and ten cm (Figure 2-1; 19), which share a common focal point, were used to increase the area of the excitation beam. The excitation light was reflected by a quad-band beam splitter (Chroma, USA, ZT405/488/561/647rpc-UF2), (Figure 2-1; 7) that reflects the incoming excitation light of all lasers but allows the emission light to pass. The emission light of the sample was collected by the objective (Olympus, Japan, UAPON 100xOTIRF) (Figure 2-1; 4) and filtered depending on the emission wavelength by different bandpass filters (Semrock, USA, 525/45 BrightLine HC, for 488 nm; Chroma, USA, 605/50 ET for 561 nm and Chroma, USA, 700/75 ET for 661 nm). All this bandpass filters were mounted in a motorized filter wheel (Thorlabs, USA, FW102C), (Figure 2-1; 13). An additional notch filter (Thorlabs, USA, NF658-26), (Figure 2-1; 8) was used to block the excitation light of the 661 nm laser. The emission light passes a slit with a width of 3.4 mm (Figure 2-1; 11) which was placed at the focal point of the tube lens with a focal length of 18 cm (Figure 2-1; 9) and cropped the emission light to match exactly the size of half the camera chip. For 3D STORM using astigmatism
a cylindrical lens with a focal length of 100 cm (Thorlabs, USA, LK10002RMA) (Figure 2-1; 9) can be placed in the beam path. The magnification of the emission light was increased by a factor of 6/5 using a telescope lens system with a focal length of 25 and 30 cm (Figure 2-1; 12, 15 and 16). The emission light was split into two channels using a dichroic beam splitter (Chroma, USA, 690 DCXR), (Figure 2-1; 14). The dichroic mirror splits the emission light at a wavelength of 690 nm. Finally, both channels were detected on the same chip of an EMCCD camera (Andor, UK, iXon Ultra 897) (Figure 2-1; 18).

The active focus stabilization was realized using an infra-red diode (Wave Spectrum, China, 785 nm), (Figure 2-1; 1) that was coupled into the excitation beam path by a dichroic filter (Chroma, USA, T770dcspxxt), (Figure 2-1; 6) and reflected at the sample. The reflected beam passing a movable mirror (Figure 2-1; 3) was detected by a quadrant diode (Laser Components, Germany) (Figure 2-1; 2). The signal of the quadrant diode was further processed by a board (Laser Components, Germany, lc-301 dqd) which fed the control unit (Physik Instrumente, Germany, E709.CRG) of the piezo controller (Physik Instrumente, Germany, P-726.1CD), (Figure 2-1; 5).

2.4.3 Stage
The heart of the custom-built microscope is the microscope stage (Figure 2-2; yellow and Figure 2-3). All parts of the microscope stage are modular designed which makes exchanging of components easy. For example, the holder for the dichroic mirror at the filter turret (Figure 2-4) can be changed or the scaffold on top of the microscope body (Figure 2-5) can be replaced so that another xy-stage can be used.
The microscope stage consists of the filter turret (Figure 2-4) microscope body (Figure 2-5). The dichroic mirrors which are used to couple in the excitation light and the light of the IR diode which is used for the focus stabilization system, are mounted on the triangle shaped filter mounts.

![Figure 2-4 Sketch of the filter turret. The dichroic mirrors used to couple the excitation beam and the IR beam for the focus stabilization into the objective are mounted here.](image)

The xy-stage and the piezo positioner are mounted on the microscope body. The piezo positioner is mounted on a cylinder with a fine thread on the outside. By turning the piezo positioner the focal point can be coarsely adjusted.
Figure 2-5 Sketch of the microscope body. XY-stage (not shown) is mounted on top of the microscope body (XYM). The piezo positioner holding the objective is mounted in a cylinder with threads on the inside and outside (OM).

2.4.4 Active focus stabilization

The active focus stabilization system was developed to correct for sample drift that occurs along the z-axis. This is particularly important to collect photons from the same physically defined location. The active focus stabilization system as used from Fruh et al.\textsuperscript{110} was implemented. As it is the most complicated part of the microscope, it will be described in greater detail in this section. The focus stabilization ensures a fixed distance between the objective and the upper glass surface, where the sample is located which results in a constant focus position. This is realized by detecting the position of an infrared (IR) beam that is coupled into the objective and reflected back from the glass surface. The IR beam is coupled into the objective in parallel to the axis of the objective but shifted from the center. Due to this shift, the IR beam exits the objective at a certain angle. If this angle is large enough, total reflection occurs at the interface between the upper surface of the glass slide and the buffer solution. The reflected IR beam shows a displacement compared to the incoming beam which depends on the distance between the objective and the upper side of the glass slide. This process is illustrated in Figure 2-6. The position of the reflected beam is measured using a quadrant diode. The signal of the quadrant diode is fed to the control
unit of the piezo positioner. If the feedback loop is active, the piezo controller alters the position of the objective, to keep the signal of the IR beam at the center of the quadrant diode. The focal position can also be changed when the feedback loop is active. To this end, a movable mirror was installed in the path of the reflected IR beam. Moving the mirror, also moves the IR beam position on the quadrant diode. Since the beam was displaced from the center, the control unit of the piezo controller shifts the position of the objective resulting in a different focal position.

![Diagram of active focus stabilization](image)

Figure 2-6 Principle of the active focus stabilization. An IR beam is coupled into the objective in parallel but displaced to the central axis. Due to the shift, the IR beam exits the objective at an angle. The beam is reflected but is now displaced compared to the incoming beam. A z-shift of the sample would lead to a displacement of the reflected IR beam. Note that this sketch illustrates the principle of the focus stabilization system, the angles and dimensions of the depicted objects are not shown in the right proportion.

### 2.4.5 Used parts

This section lists all parts that were used building the microscope. The numbers in Table 2-1 correspond to the numbers in Figure 2-1.

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## Materials and methods

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<td>AC508-180-A, Thorlabs</td>
</tr>
<tr>
<td>10</td>
<td>Cylindrical lens, focal length 1000 mm</td>
<td>LJ1516RM-A, Thorlabs</td>
</tr>
<tr>
<td>11</td>
<td>Slit with 3.4 mm width</td>
<td>Custom made</td>
</tr>
<tr>
<td>12</td>
<td>Lens, focal length 250 mm</td>
<td>AC508-250B, Thorlabs</td>
</tr>
<tr>
<td>13</td>
<td>Motorized Filterwheel, 6 one inch filters</td>
<td>FW102C, Thorlabs</td>
</tr>
<tr>
<td>14</td>
<td>Dichroic mirror 690DCR</td>
<td>F33-692, Chroma</td>
</tr>
<tr>
<td>15</td>
<td>Lens, focal length 300 mm</td>
<td>AC508-300B, Thorlabs</td>
</tr>
<tr>
<td>16</td>
<td>Lens, focal length 300 mm</td>
<td>AC508-300B, Thorlabs</td>
</tr>
<tr>
<td>17</td>
<td>Right Angle Prism 40 mm</td>
<td>PS912, Thorlabs</td>
</tr>
<tr>
<td>18</td>
<td>Camera iXon Ultra</td>
<td>DU-897U-CS0-#BV, Andor</td>
</tr>
<tr>
<td>19</td>
<td>Lens-system, focal length 10 and 100 mm</td>
<td>AC080-010-A and AC254-100-A, Thorlabs</td>
</tr>
<tr>
<td>20</td>
<td>Beam splitter 590dcxr 25.5 * 36 * 1 mm</td>
<td>NC316551, Chroma</td>
</tr>
<tr>
<td>21</td>
<td>661 nm Coherent Cube laser 100 mW</td>
<td>141667501 (not produced any more), Coherent</td>
</tr>
<tr>
<td>22</td>
<td>Clean-up filter ZET 660/20</td>
<td>F49-660, AHF</td>
</tr>
<tr>
<td>23</td>
<td>Obis 561nm LS 100 mW</td>
<td>1253302, Coherent</td>
</tr>
<tr>
<td>24</td>
<td>Beam splitter 525 DCXRU</td>
<td>F33-526, AHF</td>
</tr>
<tr>
<td>25</td>
<td>Beam splitter ZT442rdr 25.5 * 36 *1 mm</td>
<td>F48-442, AHF</td>
</tr>
<tr>
<td>26</td>
<td>Cube 405-100C, 405nm100mW</td>
<td>1170506 (not produced any more), Coherent</td>
</tr>
<tr>
<td>27</td>
<td>Obis 488nm LS 100 mW</td>
<td>1226420, Coherent</td>
</tr>
</tbody>
</table>

### Additional parts

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>Emission filter 525/45 BrightLine HC, used with 488 excitation Ø 25mm</td>
<td>F37-521, AHF</td>
</tr>
<tr>
<td>NA</td>
<td>Emission filter 605/50 ET Bandpass, used with 560 excitation Ø 25mm</td>
<td>F49-605, AHF</td>
</tr>
<tr>
<td>NA</td>
<td>Notch filter NF658-26 used with 660 excitation</td>
<td>NF658-26, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>Digital Piezocontroller E-709.CRG</td>
<td>NA, Physik Intrumente</td>
</tr>
<tr>
<td>NA</td>
<td>Board that processes the output from the quadrant diode and feeds the Piezocontroller LC-301 DQD</td>
<td>NA, Laser Components</td>
</tr>
<tr>
<td>NA</td>
<td>Mounts for beam splitters 1 inch</td>
<td>KM100S, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>Kinematic prism mount</td>
<td>KM100PM/M, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>Lens mount 1 and 2 inch</td>
<td>LM1XY/M and LM2XY/M, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>90 degree flip mount</td>
<td>TRF90/M, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>Mounts for 1 and 2 inch mirrors</td>
<td>KM100 and KM200, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>1 and 2 inch protected mirrors</td>
<td>PF10-03-P01 and PF20-03-P01, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>45 degree elliptical mirror mount</td>
<td>H45E1, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>Elliptical coated mirror</td>
<td>BB1-E02, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>12.7 mm steel posts</td>
<td>TR75/M, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>Magnetic post holder 100 mm</td>
<td>PH100E/M, Thorlabs</td>
</tr>
<tr>
<td>NA</td>
<td>XY-Stage</td>
<td>Designed by Dr. Jonas Ries and manufactured by SmarAct company, SmarAct</td>
</tr>
</tbody>
</table>
2.5 Models and parameters for simulations

This section describes how the models for the simulations were created and which parameters were used for the simulation.

Default parameters for the simulation are shown in Table 2-2. If not stated otherwise these parameters were used for all simulations.

Table 2-2 Default parameters used for simulations with SuReSim

<table>
<thead>
<tr>
<th>Parameter:</th>
<th>Values:</th>
<th>Description:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeling efficiency</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>On-off duty cycle</td>
<td>0.0005</td>
<td>From Dempsey\textsuperscript{60} for Alexa 647</td>
</tr>
<tr>
<td>Recorded frames</td>
<td>10000</td>
<td></td>
</tr>
<tr>
<td>Binding angle</td>
<td>90°</td>
<td></td>
</tr>
<tr>
<td>Sigma of angular distribution</td>
<td>0°</td>
<td></td>
</tr>
<tr>
<td>Allow bleaching</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>Background label</td>
<td>0 μm\textsuperscript{3}</td>
<td></td>
</tr>
<tr>
<td>Reproducible output</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Direct simulation</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Localization precision (x,y)</td>
<td>12 nm</td>
<td>Based on localization precision estimated from the measurement</td>
</tr>
<tr>
<td>Localization precision (z)</td>
<td>40 nm</td>
<td></td>
</tr>
<tr>
<td>Constant localization precision</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>Detection efficiency</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

2.5.1 Actin in the synaptic vesicle cluster

Models for actin were created based on EM tomograms of the SV cluster of the AZ. In these tomograms filaments that have a diameter up to approximately 10 nm were contoured using IMOD software suite\textsuperscript{112}. Afterward the lines were exported in the WIMP file format.

For the simulation of actin within the SV cluster default parameters shown in Table 2-2 and the parameters shown in Table 2-3 were used. For the Illustration in Figure 3-36 a label-epitope distance of 16 nm was used.

Table 2-3 Parameters used for the simulation of F-actin filaments within the SV cluster.

<table>
<thead>
<tr>
<th>Parameter:</th>
<th>Values:</th>
<th>Description:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitope density</td>
<td>0.36 nm\textsuperscript{1}</td>
<td>One subunit per 2.76 nm\textsuperscript{113}</td>
</tr>
<tr>
<td>Labeling efficiency</td>
<td>10%, 100%</td>
<td></td>
</tr>
<tr>
<td>Localization precision (x,y)</td>
<td>12 nm, 4 nm, 1 nm</td>
<td></td>
</tr>
<tr>
<td>Localization precision (z)</td>
<td>40 nm, 8 nm, 1 nm</td>
<td></td>
</tr>
<tr>
<td>Radius of filaments</td>
<td>5 nm</td>
<td></td>
</tr>
<tr>
<td>Label epitope-distance</td>
<td>0.6 nm/16 nm</td>
<td>Phalloidin\textsuperscript{114}/ Primary and secondary antibody\textsuperscript{115}</td>
</tr>
<tr>
<td>Fluorophores per label</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Constant localization precision</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>Detection efficiency</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

2.5.2 Synaptic vesicles

Based on EM reconstructions\textsuperscript{116} vesicle centers were determined in 3D. The diameters of synaptic vesicles are approximately 40 nm. Using the centers and the fixed radius, the surfaces of the vesicles were approximated by small triangles resembling a sphere and saved in the NFF file format.

For the simulation of the synaptic vesicles the parameters listed in Table 2-4 were used. For the epitope density 31.5 copies per vesicle and a radius of 20 nm were assumed.
Table 2-4 Parameters used for the simulation of synaptic vesicles.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values:</th>
<th>Description:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitope density</td>
<td>0.00626 nm⁻²</td>
<td>31.5 copies per vesicle (r = 20 nm)⁹⁵</td>
</tr>
<tr>
<td>Label-epitope distance</td>
<td>16 nm</td>
<td>Primary and secondary antibody¹¹⁵</td>
</tr>
<tr>
<td>Fluorophores per label</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Constant localization</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>precision</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detection efficiency</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

2.5.3 Microtubules

For the 3D model used in Figure 3-31, two filaments were modeled in 2D using the editor of SuReSim. Afterwards the z values were altered to match the z distance of the two strands of microtubules that was determined by exploring the experimental data using VISP¹¹⁷. The direct simulation mode and the parameters as given in Table 2-5 were used.

Table 2-5 Parameters used for the direct simulation of microtubules.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values:</th>
<th>Description:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitope density</td>
<td>1.625 nm⁻¹</td>
<td>13 subunits per 8 nm¹¹⁸</td>
</tr>
<tr>
<td>Radius of filaments</td>
<td>12.5 nm</td>
<td></td>
</tr>
<tr>
<td>Label-epitope distance</td>
<td>16 nm</td>
<td>Primary and secondary antibody¹¹⁵</td>
</tr>
<tr>
<td>Labeling efficiency</td>
<td>10%, 50%, 100%</td>
<td></td>
</tr>
<tr>
<td>Fluorophores per label</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Constant localization</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>precision</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detection efficiency</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

2D models of microtubules were created using the editor of SuReSim by importing the reconstruction of STORM measurement of microtubules and modeling the structure. For the simulations shown in Figure 3-32, Tiff stacks were simulated using the parameters as shown in Table 2-6.

Table 2-6 Parameter used for the Tiff stack simulation of microtubules.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values:</th>
<th>Description:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitope density</td>
<td>1.625 nm⁻¹</td>
<td>13 subunits per 8 nm¹¹⁸</td>
</tr>
<tr>
<td>Radius of filaments</td>
<td>12.5 nm</td>
<td></td>
</tr>
<tr>
<td>Label-epitope distance</td>
<td>16 nm</td>
<td>Primary and secondary antibody¹¹⁵</td>
</tr>
<tr>
<td>Fluorophores per label</td>
<td>1.6</td>
<td>Determined from experiment</td>
</tr>
<tr>
<td>Constant localization</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>precision</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detection efficiency</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Direct simulation</td>
<td>false</td>
<td></td>
</tr>
<tr>
<td>Mean number of photons</td>
<td>1320/1802/2709</td>
<td></td>
</tr>
<tr>
<td>Exposure time</td>
<td>30 ms</td>
<td></td>
</tr>
<tr>
<td>Minimal Photon Count</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>Mean blinking duration</td>
<td>30 ms</td>
<td></td>
</tr>
<tr>
<td>Pixel to nm ratio</td>
<td>133 nm</td>
<td></td>
</tr>
<tr>
<td>Frame rate</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Dead time</td>
<td>0 ms</td>
<td></td>
</tr>
<tr>
<td>Readout noise</td>
<td>22 DN</td>
<td></td>
</tr>
<tr>
<td>Constant offset</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>EM gain</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>
Quantum efficiency | 1 |
Electrons/DN | 4.81 |
Windowsize for PSF rendering | 10 px |
Empty pixels on rim | 5 |
PSF model | 2D |
Numerical aperture | 1.45 |
Wavelength | 647 nm |
Defocus | 800 nm |
Focus | 400 nm |
Ensure single PSFs | NO |
Distribute PSFs | YES |

### 2.5.4 Mitochondria

The electron microscopy model of mitochondria that was used for the simulation was published by Marsh et al.\textsuperscript{119}. The epitope density was estimated based on published values for the number of TOM20 clusters on mitochondria and the average number of epitopes per cluster\textsuperscript{120}. The direct simulation workflow was used with the parameters specified in Table 2-7.

**Table 2-7 Parameters used for the simulation of mitochondria.**

<table>
<thead>
<tr>
<th>Parameter:</th>
<th>Values:</th>
<th>Description:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitope density</td>
<td>0.001 nm$^2$</td>
<td>(~10$ TOM20 per Cluster, (~100$ cluster per $\mu m^2$\textsuperscript{120}</td>
</tr>
<tr>
<td>Label-epitope distance</td>
<td>16 nm</td>
<td>Primary and secondary antibody\textsuperscript{115}</td>
</tr>
<tr>
<td>Fluorophores per label</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Constant localization precision</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>Detection efficiency</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

### 2.5.5 Actin in erythrocytes

For the simulation of subcortical F-actin in the membrane of erythrocytes another published model, determined by cryo-electron tomography\textsuperscript{121} was used. The model shows actin at the membrane of an erythrocyte. The direct simulation workflow with the parameters given in Table 2-8 was used.

**Table 2-8 Parameters used for the simulation of actin in erythrocytes.**

<table>
<thead>
<tr>
<th>Parameter:</th>
<th>Values:</th>
<th>Description:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitope density</td>
<td>0.0115 nm$^2$</td>
<td>1 binding site per 2.76 nm, radius 5 nm.\textsuperscript{122-124}</td>
</tr>
<tr>
<td>Label-epitope distance</td>
<td>0.6 nm</td>
<td>Phalloidin\textsuperscript{114}</td>
</tr>
<tr>
<td>Fluorophores per label</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Constant localization precision</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>Detection efficiency</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

### 2.5.6 Nuclear pore complex

The ground truth model for nup133 of the nuclear pore complex was created as a binding site model. First a single pore was simulated as an octagon with diameter of 106 nm and an edge length of 40 nm\textsuperscript{125}. A four-fold multiplicity for the epitopes was simulated. Multiple nuclear pore complexes were simulated at multiple positons. For each position a random rotation of the epitopes was introduced.
### Table 2-9 Parameters for the simulation of nup133 protein.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeling Efficiency</td>
<td>43%</td>
<td>SNAPf-tag</td>
</tr>
<tr>
<td>Label-epitope distance</td>
<td>2 nm</td>
<td>SNAPf-tag</td>
</tr>
<tr>
<td>Fluorophores per label</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Constant localization precision</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>Detection efficiency</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>
3 Results

This chapter summarizes the results I made during my PhD. In the first part, the 3D spectral demixing STORM microscope I assembled and partly designed is briefly described (see Materials and Methods for more detail), followed by a description of the post-processing software I developed. The last part will showcase the limitations of STORM which led to the development of a software package called SuReSim that helps to simulate the expected outcome of SMLM experiments.

3.1 dSTORM microscope and associated software

In order to have an optimized setup to answer given experimental questions, I partly designed and constructed a STORM microscope and developed a controlling software for easy handling of the individual components. The scope was built to enable 2D and 3D dSTORM imaging using spectral demixing to avoid chromatic aberration. Its active focus stabilization reduces focus drift and provides the needed spatial and axial resolution to investigate the molecular composition of the presynaptic nerve terminals.

This section will give a brief summary over the components of the microscope, it will show the validations performed to ensure the performance of the scope and a detailed description of its control software.

3.1.1 Summary of the microscopes components

A detailed description of the microscope can be found in section 2.4. Figure 2-1 shows an overview of the components of the microscope. In the upper part, the active focus stabilization system is shown, consisting of an infrared diode (1) a quadrant diode (2) and a movable mirror (3). The objective (4) is mounted on a piezo-driven stage (5). The infrared light is coupled into the objective by a dichroic mirror (6). The excitation light is reflected onto the sample using a second dichroic mirror (7). The emission path consists of a notch filter (8) that blocks the 661 nm excitation laser (21). Followed by the tube lens (9) and a cylindrical lens (10) that can be used to introduce astigmatism to the PSF. At the focal point of the tube lens a slit (11) is placed, followed by another lens (12) that creates a parallel beam again. Different emission filters to block excitation light are placed in a filter wheel (13). The dichroic (14) splits the emission light into two channels that are focused by the two lenses (15 and 16) on the camera (18). One of the two beams is reflected at the inner edge of a prism (17). The excitation light is widened by two lenses forming a telescope (19). The 661 nm laser beam (21) is merged with all the other lasers at the last dichroic (20) of the excitation path. A clean up filter (22) is used for the 661 nm laser (21) to reflect all other wavelengths and transmit only the primary wavelength. The 561 nm laser (23) is merged with the UV-laser (26) and the 488 nm laser (27) using two dichroic mirrors (24 and 25).

3.1.2 Validation of the microscope

This section shows the validation of the performance of the microscope setup. The pixel size, the alignment of both channels at the camera chip, the achievable localization precision and the active focus stabilization were validated.

3.1.2.1 Validation of pixel size

The pixel size determines the relation between sizes of objects in the sample and the number of pixels they span in the recorded image. It is determined by the size of the camera chip, the number of pixels on the camera chip, the objective and tube lens and by the magnification which is introduced by a telescope lens system. With a hundred-fold magnification given by the
objective and the tube lens and a pixel size of 16 μm of the camera chip, the pixels size of the microscope should theoretically be 160 nm. The image is magnified by the telescope system by a factor of 6/5, resulting in a theoretical pixel size of 133.3 nm.

To measure the actual pixel size achieved by the microscope a test sample that consists of a fine grating with a distance of 10 μm between the individual bars was placed in the focus of the objective. The sample was illuminated with almost parallel light. The resulting image is shown in Figure 3-1. The actual pixel size of 132 nm can be calculated from the known distance between the bars of 60 μm and the measured distance of 455 pixels. This image was taken before the demixing dichroic mirror was placed in the emission beam path, therefore there is only one channel visible on the camera chip.

![Figure 3-1 Test image of a grating. The distance between individual bars measures 10 μm.](image)

3.1.2.2 Alignment of both channels

After placing the dichroic mirror into the beam path, the two channels had to be aligned on the camera chip. Additionally, the two lenses placed between dichroic and camera had to be accurately aligned for both channels. Incorrect placement of these lenses would lead to aberrations. An example for incorrect and correct alignment is given in Figure 3-2. For both images, fluorescent beads were placed on a glass cover dish and imaged. The images of both channels were manually shifted to overlap. In Figure 3-2 (a), strong distortions between both channels can be seen. In Figure 3-2 (b), small distortions are still present but can be neglected and will be compensated by the post-processing software.
3.1.2.3 Evaluation of the localization precision

The localization precision achievable in STORM depends on different parameters. The mean photon output number of a fluorophore, the proper illumination, the exposure time, the filters and the reconstruction software used - all these factors influence the precision of the estimated fluorophore position.

To determine the localization precision that can be achieved using the new microscope measurements on microtubules in COS-7 cells, stained with Alexa Fluor 647 were performed. Both the lateral localization precision for 2D and for 3D measurements were determined using the nearest neighbor analysis (NeNA) method\(^5\) which estimates the localization precision by analyzing the lateral deviation of blinking events distributed over consecutive frames. Additionally two theoretical measures as reported by Thompson et al.\(^5\) and Mortensen et al.\(^4\) that take parameters like the photon number and noise into account were used to determine the localization precision. All three methods were performed using the LAMA software package\(^1\). Only the measurements of the left channel were reconstructed by RapidSTORM, because no demixing was necessary for the reconstruction of only one kind of fluorophore. The parameters for the Thompson and Mortensen localization precision analysis were estimated based on the PSF width and background intensity present in the raw data. The results summarized in Table 3-1 show that the estimated localization precision matches the theoretical localization precision calculated using the Mortensen method very well. Mortensen et al. state in their paper\(^4\) that Thompson et al.\(^5\) underestimated the actual localization precision, which is also reflected by the acquired data in this thesis.

The estimated lateral localization precision of 6.5 nm for 2D and 9.4 nm for 3D measurements is worse than it would be theoretically possible using the Alexa Fluor 647 dye as the intensity of the fluorophores is distributed over two channels and only one channel is used for reconstruction. The lower value for the 3D measurement can be explained by the effects induced by astigmatism which increases the PSF width compared to the 2D measurements and thus increases the influence of noise.
3.1.2.4 Validation of the active focus stabilization system

To validate the stability of the focus stabilization, fluorescent beads were placed on the glass surface of a glass bottom dish. The focus was adjusted manually to focus on the beads. The feedback loop was engaged and a measurement was performed. The same procedure was repeated without active focus stabilization. The result and comparison of one of these measurements is shown in Figure 3-3. The reconstruction of the same bead once with and once without active focus stabilization is shown as a function over time. The fitted z-values for both the activated and deactivated focus stabilization were smoothed with a moving average filter with a width of forty seconds to compensate for the localization error. The first twenty seconds and the last twenty seconds are not shown because they could not be smoothed due to the moving average filter. The active focus stabilization strongly limits the variations of the reconstructed z-values.

![Figure 3-3](image)  
**Figure 3-3** Active focus stabilization vs uncompensated focus drift. The blue line shows a bead position with engaged focus stabilization, the red line without active focus stabilization. The change in the focal position over half an hour is much higher without the active focus stabilization. For this graph, the bead position was smoothed by a moving average filter to smooth the localization uncertainty present in the fitted bead position.

3.1.3 Microscope control software

For each component the respective software of the manufacturer could be used to control. The downsides of the use of the manufacturers software are that many different software solutions have to be used and that there is no possibility to connect the functionality of different components. For example, it is not possible to shut down the laser once the image acquisition is finished. To avoid the above mentioned problems, a custom-made software was implemented to control almost all components of the microscope. This was realized using the open-source software Micro-Manager\textsuperscript{107}, a plug-in for the widely used open-source image processing platform ImageJ\textsuperscript{129}. Micro-Manager was developed in order to control microscopes, cameras and other

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Table 3-1 Calculated localization precision in x and y direction for 2D and 3D measurement of microtubules.

<table>
<thead>
<tr>
<th></th>
<th>2D</th>
<th>3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nearest neighbor analysis:</td>
<td>6.5 nm</td>
<td>9.4 nm</td>
</tr>
<tr>
<td>Thompson:</td>
<td>5.8 nm</td>
<td>7.5 nm</td>
</tr>
<tr>
<td>Mortensen:</td>
<td>6.8 nm</td>
<td>8.6 nm</td>
</tr>
</tbody>
</table>
components associated with microscopy and to provide easy access to the functionality of these components. Micro-Manager and ImageJ both offer application programming interfaces (API) for JAVA, thus making it easy to write custom JAVA graphical user interfaces (GUI). The rationale behind the development of a custom GUI that combines access to all components as stated above is that it enables different components to communicate with each other and ensures a user-friendly operation of the microscope.

### 3.1.3.1 Graphical user interface

Figure 3-4 shows the design of the GUI for the microscope controlling software. The different options of the software will be discussed in greater detail in the following section 3.1.3.2. Briefly, a live preview of the frame currently captured by the camera is shown in the upper left corner (Figure 3-4). The display is updated whenever a new frame is captured. On the right, the laser control panel can be found. Currently, only the laser emitting 661 nm light and the UV laser emitting light with a wavelength of 405 nm are implemented in the GUI. Below the laser control panel, a section with multiple tabs shows the settings for image acquisition, selection of the region of interest (ROI) and online reconstruction (Figure 3-4; for the respective tab contents see expanded panel on the right). The panel to control the output directory (destination where the acquired images will be saved to) is located directly below these tabs. On the lower right, a monitor shows the current piezo stage position. In the lower left corner, the piezo stage control panel with options to set the position of the piezo stage and an option to automatically capture calibration stacks as needed for the 3D astigmatism approach are located.

![Figure 3-4 Graphical user interface of the microscope controlling software. On the right, the contents of the three available tabs are shown.](image-url)
3.1.3.2 Elements of the microscope control software

3.1.3.2.1 Live preview window
In the upper left corner a live preview of the current frame captured by the camera can be seen. Both channels split by the dichroic are visible next to each other. Below the preview window there are two buttons which can be used to increase and decrease the zoom-factor. This zoom only affects the way the images of the camera are displayed. It neither alters which part of the images is stored during image acquisition, nor the magnification of the captured images. The scaling factor is a potency of two, meaning that each dimension of the original image is stretched by a factor of 1, 2, 4, 8, and so on. That way no interpolation is needed since an original pixel is either displayed as 1, 4, 16, 64, ... pixels in the preview window.
Furthermore, to facilitate perception by the human visual system, the contrast of the image can be kept constant by selecting the checkbox “lock values”. This disables the dynamic range which is activated by default. A minimal and a maximal intensity value can be set. All intensity values equal to or larger than the upper bound will be displayed with the highest intensity (white). Likewise, all pixels with an intensity value smaller than the minimal value will be set to be black. The dynamic range estimates the intensity range that is needed. Therefore the pixel with highest and lowest intensity are used as limits to which each ten percent of the total intensity range is added or subtracted, to find the upper and lower intensity bounds. The additional ten percent margin is added to prevent flickering. The borders are only updated when a pixel is brighter than the upper bound or has a lower intensity than the lower bound. Again, new bounds are determined in the way described above.
On the right, the smallest and highest pixel intensity is displayed as well as the average of all pixels. These numbers are updated for each frame taken.

3.1.3.2.2 Laser control panel
The laser control panel can be found on the upper-right part of the GUI. Currently, only the 661 nm and the 405 nm laser are supported by the GUI. The intensity of the laser can either be set using the vertical slider or the text box. The command to change the laser intensity is send to the laser by pressing the on/off-button. Until then, the laser intensity stays at the previously set value.
At the bottom of the laser control panel, the desired number of blinking events per frame can be set. Once a number is set and the checkbox “Enable UV Laser Control” is activated, the UV laser intensity is increased each time the average number of blinking events calculated on the last 10 frames becomes lower than the entered value.
To calculate the number of blinking events per frame, the image of the frame is Gaussian-blurred with a sigma of one pixel. In the Gaussian-blurred image the number of local maxima is determined. Only maxima with an intensity that exceed the intensities of their neighbor pixels by at least 4 times the standard deviation of the intensities of the filtered image are considered. For the blurring and the determination, of the maximum the ImageJ functions “getMaxima” from the “maxiumFinder” function and the “GaussianBlur” function were used.

3.1.3.2.3 Camera settings
The camera settings tab (see Figure 3-4) offers access to the camera gain, the electron multiplying (EM) gain as well as the exposure time in milliseconds, the target temperature of the CCD chip, the shutter position and the number of frames that are to be captured. With the checkbox “Frame
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The user can switch between the frame transfer image acquisition mode and the normal mode. The exposure time chosen takes immediate effect during the live preview but is ignored when altered after an image sequence acquisition has been started.

3.1.3.2.4 ROI settings
The microscope control GUI (see Figure 3-4) enables to choose regions of interest that are smaller than the full frame. Since the left and the right channel show the same structures but different wavelengths, the chosen region in the left channel will also be selected in the right channel. Choosing only a certain region of the whole image might be advantageous if the structure of interest is only present in a small portion of the image. In this case, capturing only the smaller ROI would reduce the size of the stored Tiff (tagged image file format) stack.

By clicking somewhere in the left half of the preview window and keeping the left mouse button pressed, a rectangular region can be selected and will be visible in the preview image. The selected rectangle is shown in the left half of the preview window and a corresponding window of the same size and part of the sample is displayed on the right. The coordinates of the upper-left corner of the drawn rectangle will be displayed in the textboxes labeled “X:” and “Y:”. The width and height of the rectangle is displayed at the respective textboxes. All values can be altered manually. Below these four textboxes, two additional textboxes can be found. Here, the offset between the left and the right rectangle can be adjusted. This offset might be used to compensate for a shift between the left and the right channel.

Below the textboxes, the user can choose between three options: either the left, right channel or both channels can be stored. It is important to notice that in the current version of the software, the selection of ROIs does not restrict the size of the image captured by the camera and therefore does not increase the maximal frame rate for small ROIs. It only determines which part of the image is stored.

In the bottom row, there are options to show the central line between both channels, to reset the rectangle as well as an option that let the user decide whether or not the selected ROI is to be applied. This checkbox is automatically checked when a ROI is drawn.

3.1.3.2.5 Output path
The folder in which the individual measurements will be stored can either be written into the text field or set by selecting the desired folder using the “set path” button (Figure 3-4). The string entered in the text field “Measurement tag” specifies the folder name that is used for the current measurement. This tag should be altered for each new measurement. If the measurement tag is not altered, a warning will be displayed giving the options to change the measurement tag or to overwrite the previously acquired data.

The microscope control software will create a folder hierarchy within the selected folder. Two separate folders for the images from the left and right channel and one folder for the output of the reconstruction algorithm will be created. Additionally, a log file with the imaging parameters chosen for the measurement will be saved in the selected folder.

3.1.3.2.6 Live reconstruction using RapidSTORM
To increase the convenience and reduce the processing time for the acquisition of a STORM image, the reconstruction process of the raw data is started while the acquisition is still running. For this purpose RapidSTORM, a fast and freely available reconstruction algorithm is used. Whenever a complete Tiff stack is written on the hard drive, the reconstruction of this part of the
measurement is automatically started if the checkbox “Do simultaneous processing” is selected. Right next to this checkbox the option to reconstruct the data using the astigmatism approach for additional information along the z-axis is available. If the astigmatism approach is used the path to a calibration file needs to be provided. The path to this file has to be specified in the “Calibration File” text field. The user can also choose between two different thresholding methods and the corresponding parameter. If live reconstruction is enabled, new folders are created in the selected output folder to organize the results of the reconstruction algorithm.

The button “Recalculate Everything” repeats the reconstruction process for the specified folder and can be used to recalculate a whole measurement with altered parameters. Due to the disabled command line option for RapidSTORM under windows a complicated workaround was used in order to perform the live reconstruction. It will be explained in more detail in section 3.1.3.3.3.

3.1.3.2.7 Camera control
The camera control consists of five buttons that start and stop the live preview or image acquisition and capture a widefield image. By starting the live preview the camera will take images with the specified frame rate and these images will be displayed in the preview window. These images are not stored and this mode is used to check the parameters and find the proper focal position before starting image acquisition. This mode is ended by the “Stop Live Preview” button.

The “Start Acquisition” button will start the image acquisition of a series of images with the parameters specified in the camera settings tab. The image acquisition can be ended prematurely by clicking the “End Acquisition” button; otherwise the image acquisition will end when the selected number of frames was recorded. If the size of all images of an image sequence exceeds 2.6 gigabyte, the image sequence is broken into multiple parts with a maximal size of 2.6 gigabytes. This is done because RapidSTORM can only process Tiff stacks with a maximal size of four gigabytes. If the uncropped channel is recorded this limit corresponds to 11,000 images per Tiff stack.

The “Capture Widefield Image” button can be used to save an image with the currently chosen parameters into the selected measurement folder.

3.1.3.2.8 Piezo stage control
The objective is mounted into a piezo-controlled stage. The position of the stage can either be set or a feedback loop, as described in the previous section, can be used. On the lower right corner of the GUI (Figure 3-4) a monitor that shows the current piezo stage position is located. When the feedback loop is disengaged, the position of the piezo stage is correctly visualized. Once the loop is engaged, the reported values from the stage range from -1 to +1 and do not represent the current piezo position but processed feedback from the quadrant diode.

The piezo stage control panel is placed in the lower left corner of the GUI. When the feedback loop is disengaged, the position can be directly entered into the text field in the upper left corner of the control panel. The position can also be altered using the arrow keys of the keyboard if the text field is selected. The step size of single steps can be adjusted with the help of the selection box to the right. In the upper right corner of the piezo control panel, a checkbox entitled “Focus Lock” that engages the feedback loop is located.

The piezo control panel can also be used to create calibration stacks for the astigmatism approach. In this case, a preferably bright, fluorescent bead is imaged at different focal positions. The lower and upper bound of the calibration stack can be set comfortably by pressing the “Set
Lower Bound” or “Set Upper Bound” buttons, which will insert the current piezo stage position into the text field for the respective bound. Also, the step size for the calibration stack can be set. Once the “Record Stack” button is pressed, the microscope control software will capture images of increasing z-positions with the given step size and store them in the indicated measurement folder.

3.1.3.3 Technical realization

3.1.3.3.1 Background for the Micro-Manager plugin
A detailed instruction on how to write plugins for Micro-Manager can be found on the Micro-Manager website (https://www.micro-manager.org/). Briefly, to build a plugin for Micro-Manager, it is necessary to create a Java class that implements the Micro-Manager MMPlugin interface. This interface gives access to a core object which allows control over all devices loaded from Micro-Manager. The plugin has to be compiled into a .class or .jar file and must be saved in the “mmplugins” directory of the installation path of Micro-Manager. It is important to compile the Java class with the appropriate Java Runtime Environment, currently Java 1.6.

3.1.3.3.2 Program structure of the microscope control software
The plugin class is called “MainClass”. When the Microsoft control software is started, its “setApp” method is executed, which initializes the GUI of the microscope control software. The “MainFrame” class creates the window of the microscope control software. In the “MainFrame” class, all control panels for the individual components are initialized. The communication between different hardware components is also managed via the “MainFrame” class. To maintain the response of the plugin for each process, like the update of the temperature of the camera, the piezo control monitor or the image acquisition independent threads are used.

3.1.3.3.3 Use of Python for the live reconstruction
The live reconstruction of the acquired Tiff stacks employs RapidSTORMs command line feature. Using the command line feature, RapidSTORM is executed without involving its GUI. All necessary parameters like the threshold value or the in- and output filenames are directly specified. Unfortunately, the current version of RapidSTORM 3 does not support the command line feature for the Windows operating system. As an alternative the programming language Python was used. The command line version of RapidSTORM can be used from Python on any operating system. To realize a live reconstruction for Windows, a Python script is created that executes the command line version of RapidSTORM with the selected parameters. Then the microscope control software executes the command line version of Python and lets Python execute the Python-script, which in turn runs RapidSTORM, which then reconstructs the current chunk of data.

3.2 Post-processing software
After reconstructing the acquired images a list containing information about the estimated location, the intensity and the frame of each fitted blinking event is generated. This reconstructed data needs further processing, for example, to correct for sample drift that might have occurred during image acquisition or to perform the demixing steps. For this purpose, I wrote a post-processing software which can process data from two common reconstruction algorithms, RapidSTORM and ThunderSTORM. The aim of the development of the post-processing software was to create a flexible and easily usable tool compatible with the STORM image
acquisition workflow. A custom-made GUI (Figure 3-5) provides user-friendly access to the post-processing options.

3.2.1 Overview

The software uses individual modules which will be explained in more detail in the next section. A typical STORM workflow can be seen in Figure 3-5. Modules can be chosen from three categories on the left side of the GUI. In the central column the current order of the post-processing modules is displayed. All the modules can be dragged to another position to alter the order of post-processing steps. By clicking the red “X”, individual modules can be deleted from the list of selected modules. If a module is selected its parameters are shown on the right and can be altered.

In general, there are three types of modules: import modules that load a list of localizations from the reconstruction algorithm into the post-processing software, processing modules that alter the localizations and output modules that save images or text files based on the current localization list.

Workflows that were created (order of modules and the parameters set) can be saved to be loaded into a new session. There is also a list of example workflows available that can be altered.

Figure 3-5 Graphical user interface of the post-processing software.
The post-processing software loads the list of localizations created by the reconstruction software and represents this list of localizations as list in which each localization is an object with the following properties:

1. **X-value:** The distance in nm from the upper left corner in x-dimension
2. **Y-value:** The distance in nm from the upper left corner in y-dimension
3. **Z-value:** Assigned z-value from the reconstruction algorithm in nm
4. **Frame:** Frame in which the localization was detected
5. **Intensity:** Intensity in photons (only if the reconstruction software is properly calibrated)
6. **Angle:** Angle in rad of the localization in an intensity scatter plot (only used for demixed localizations)

### 3.2.2 Import modules

For the post-processing workflow, only one of the four import modules available must be chosen, first. Their function is to load one or multiple reconstructions into the post-processing software. The import modules are called “Single File Input”, “Dual-Channel Single File Input”, “Multiple File Input” and “Dual-Channel Multiple File Input”. Depending on the chosen module, either one or both channels can be imported. In addition, for each choice there is the option to import only one reconstruction per channel or to specify a folder and a pattern shared by all reconstructions of the same channel. The import of multiple files per channel is used when the image sequence of a channel exceeds the limit of four gigabytes and therefore has to be stored in multiple chunks instead of one large Tiff stack.

For each module there are similar parameters. There is the path that corresponds to the folder in which the reconstructions are located. For single file imports the filename has to be specified, for multiple file imports, a name pattern that is shared by all reconstructions which belong to one channel has to be entered. If a multiple file import module is selected all files in the selected folder that contain the pattern “settings” or “protocols” are omitted. These files are usually setting files that store the information about the reconstruction process and do not contain any localizations. Additionally, a “Basename” can be chosen. This option determines which shared root name all output files will be given.

### 3.2.3 Processing modules

Processing modules are modules that alter the current list of localizations, either by changing the parameters of single localizations (drift correction, point connection and demixing) or by removing certain localizations from the list (cropping).

#### 3.2.3.1 Drift correction

The first processing module is the drift correction module. It provides a feature based drift correction that is capable of correcting non-linear sample drift. The algorithm is based on the drift correction method published by Mlodzianoski et al.\(^ {130} \). For the drift correction the following assumptions are made:

- The sample drift is continuous but not necessarily linear, i.e. the sample does not rapidly change its position but moves continuously
- The drift within a single frame can be neglected
- All parts of the localization list show the same structure, i.e. the sample does not alter its shape during image acquisition
All of these assumptions hold true during a measurement. If the sample drift is caused by thermal effects, it would be continuous, leading to a drift of several 100 nanometers per hour. Therefore, the drift within a single frame of 30 milliseconds is negligible.

The drift correction works as follows. At first, all localizations are binned in non-overlapping chunks of a user-set size, typically 5000 frames. For each image its Fourier transformation is calculated, followed by a pairwise phase correlation of the images\(^1\) that detects the shift between the two original images (before the Fourier transformation) in the spatial domain. For the phase correlation first both Fourier transformed images are multiplied and the maximum in the resulting image is determined with a subpixel precision using a 2D Gaussian fitting algorithm. The shift of the peak relative to the center of the image corresponds to the shift of the original images in the spatial domain. Due to the image correlation the shift between the middle frame of each chunk of localizations is known. In a last step, all localizations are corrected according to the detected shift. The assumption is that the middle frame of the first chunk is not shifted and the detected shift occurred between the middle frames of the corresponding chunks. In principle, the shift would be defined by the shift between the first and the second chunk, the second and the third chunk and so on. But additional information about the shift between the first and the third chunk or the second and the fifth chunk is available due to the pairwise correlation of all chunks. The mean shift in comparison to the first chunk is calculated based on all pairs.

After that, the drift between the frame in the center of each chunk is known. To estimate the drift of any other frame, a spline interpolation is used to interpolate the drift estimates for the frames between the centers of the chunks. Using the spline interpolation, the estimated shift is subtracted from each coordinate based on its frame.

The drift correction algorithm requires three parameters as shown in Figure 3-6. The number of images per chunk determines into how many chunks the localization list is split. The smaller the chunk size, the better the performance of the drift correction for nonlinear drift. But at the same time the smaller chunk size would also reduce the precision of the shift estimation between two chunks. A chunk size of 5000 frames is a good compromise between both effects. In the second textbox the pixel size of the rendered images in the spatial domain can be specified. Smaller pixel sizes increase the precision of the shift estimation but increase the computational effort as well. The drift correction works well with values ranging between ten to twenty nanometers per pixel. Increasing the spread with which a localization is represented in the rendered image (“Sigma for drift correction”) increases the robustness of the shift estimation as well. A spread of 10 nm works well in practice.

### 3.2.3.2 Connect points

The fluorophores used for STORM measurements stay in the light-emitting state for a couple of milliseconds. The duration of the blinking event lies in the order of magnitude of the exposure time of a single frame. Thus, in most cases a blinking event is distributed over at least two frames. If the PSF is detected in consecutive frames, these localizations can be connected to decrease the localization uncertainty by averaging the position and prevent over-counting of blinking events. The assumption for this method is that the likelihood for a localization emerging in two consecutive frames originating from the same blinking event is much larger than the probability that two distinct fluorophores emitted light in close spatial and temporal proximity.
The “connect points” algorithm searches for each localization in consecutive frames for a localization that is in close proximity. The algorithm checks if the localization in the following frames lies within the distance specified in the “Maximal Tolerated Distance” parameters. A user-defined number of missing frames is allowed. If two localizations are found that fulfills both criteria of temporal and lateral proximity, the procedure of finding a localization in consecutive frames is repeated for this localization. In this way, a list of multiple related localizations that will be connected to one single blinking event is found. If no further localization can be found, a new localization is created. Its coordinates are calculated based on the coordinates weighted with the square root of the intensities; the frame of the first localization is used for the resulting localization and the sum of the intensities determines the resulting intensity. After that, all individual localizations that were merged are deleted from the localization list. Due to the instant removal of all localizations that are connected, no localization can contribute more than once.

For the “connect points” algorithm four parameters are required, as shown in Figure 3-7. The “Maximal Tolerated Distance” parameters define how much the given coordinate in the following frame might differ from the corresponding localization in the previous frame. The “Maximal Tolerated Distance in Frames” parameter defines how many frames can be omitted between consecutive localizations without interrupting the connection.

### 3.2.3.3 Spectral demixing

A key feature of the new microscope is the use of spectral demixing similar as described by Lampe et al.\(^1\). In contrast to the published algorithm where the matching relies on a known shift between the two channels, my algorithm allows both channels to be shifted in both lateral dimensions and slightly rotated relative to each other.

The photons emitted by each fluorophore that is used are split according to their wavelength and recorded in two channels on the camera. Both channels are reconstructed, resulting in two lists of localizations. Ideally these two lists contain the same localizations but with different intensities. The demixing module finds the corresponding localizations in both channels and calculates the intensity ratio which is later used by the rendering modules to assign colors. The demixing algorithm has two different parts. First of all, a good transformation that maps the second channel onto the first one is determined in an iterative manner. In a second step this best transformation, from now on called “final transformation”, is applied to all localizations of the second channel and the matching localizations are detected and stored in a separate list. An overview over the demixing workflow is given in Figure 3-8.

For the registration between both channels, an affine transformation is used. This transformation can shift, rotate and shear the positions of the second channel. A large number of localizations should have been detected in both channels and can be used to find a valid registration.

At first, three localizations from the first channel are randomly chosen. For all three localizations, a partner in the second channel is selected based on a probabilistic approach that favors proximal localizations in the untransformed second channel. Once, a partner was assigned to each point, the affine transformation that maps the partner points onto each other, is determined. This transformation is then applied to all points of the second channel. All localizations from the first

![Figure 3-7 Parameters for the connect points algorithm.](image)
and the transformed second channel are compared and the number of points in close proximity is determined. The number of matching points is a measure of how well the transformation worked and also indicates that the set of three points from both channels belonged indeed to the same blinking event.

This process is repeated multiple times and the set of points that lead to the best transformation with the highest number of matches is remembered. For transformations with the same number of matches, the set of points leading to the lowest root mean square error between the matching points is remembered. The process above is repeated for ten different frames. For each frame, a set of three points for both channels is determined. To find the final transformation, an affine transformation is calculated based on all sets of corresponding points that led to a good transformation.

The demixing is performed for chunks of frames. In that way, the algorithm is more robust against bad estimates for the final transformation as it affects only subsets of the data. The final transformation is then applied to all points of the second channel. Afterwards, again matching points, i.e. points from the first channel and the transformed second channel that lie closely together, are found within each frame. A lower limit for the intensity can be set, removing all localizations, which are darker than the specified value, from further processing.
Figure 3-8: Illustration of the demixing workflow. Ten frames are selected from the whole chunk. For each frame, an affine transformation is found. For that, three points are chosen randomly from the first channel (cyan dots). Subsequently, the corresponding points in the second channel (black dots) are selected. An affine transformation is calculated that transforms the black onto the cyan dots. The number of matches (depicted as green circles) is determined and is used as a quality measure for the transformation. This procedure is repeated multiple times for the same frame as well as for nine other frames. A “final transformation” is calculated based on the sets of points that led to a successful transformation. This “final transformation” is used to process the whole chunk. Matching points (green circles) are identified and are stored for further processing. For these points, also the intensity ratio is determined. All other points that have no partner in the other channel are removed from further post-processing (red crosses).
A new localization list is created containing only points that were found in both channels. For these points the coordinates and frames from the first channel are used, their intensity is the sum of the intensities from both channels and the angle $\alpha$ is calculated based on the intensity ratio in both channels as shown in Equation (3.1).

$$\alpha = \tan^{-1}\left(\frac{I_{\text{Ch1}}}{I_{\text{Ch2}}}\right)$$  \hfill (3.1)

The demixing steps described above are performed chunk-wise. All demixed points are added to the same list of demixed localizations.

For the demixing module five parameters can be set, as shown in Figure 3-9. Larger chunk sizes speed up the processing since the best transformation has to determined less often, but can significantly decrease the number of matches. If the checkbox “Save Paired Output” is selected, a list that contains information about the pair of matching localizations from both channels is created in the output folder. This option is mainly used for debugging or to identify a systematical error in the alignment of the two channels. In the “Number of Iterations” text field the number of attempts to find the perfect transformation for each individual frame can be specified. A higher number leads to a better transformation but at the same time increases the computational effort. The “Maximal Distance” parameter defines the tolerated lateral distance for which the points from the first and the transformed channel are considered as matching during the search for the best transformation. With the “Minimal Intensity” parameter localizations that are too dark and therefore not trustworthy can be omitted from further post-processing. The “Tolerated Error” parameter determines up to which lateral discrepancy points from the first and the finally transformed second channel are considered as matches. Both the “Maximal Distance” and the “Tolerated Error” parameters act similarly but at different steps of the demixing algorithm. It might be beneficial to choose only very well matching points during the search for the best transformation but relax this constraint when searching for matching points after the final transformation.

### 3.2.3.4 Cropping

If only part of the data is needed, for example, only the central part of the field of view containing the protein of interest, the cropping module can be used to truncate the list of localizations. The desired range of all spatial coordinates, frames and intensities can be specified. There are checkboxes available to select which input channel will be cropped.

For each property with unspecified values, the entire range is selected. It is important to note that localizations that are lost due to cropping cannot be restored in the current post-processing workflow. These localizations have to be imported again or the workflow has to be restarted.
3.2.4 Output modules

Output modules are modules that do not change the list of localizations but use it to create one of two output types, images or text files. The output is created using the current state of the localization list. Output modules are not restricted to the end of the processing workflow. For example, it might be reasonable to create an image before and after the drift correction to notice the effect.

The first output module creates an output folder at the path of the localization list. The same folder is used to store subsequently generated output.

3.2.4.1 Rendering images

One kind of output module are the image rendering modules. There are three different kinds of modules for 2D, 3D and demixed images. The task of these modules is to create and store images from the processed list of localizations. All rendering modules use a Gaussian distribution to represent individual localizations.

3.2.4.1.1 Rendering of 2D images

To create STORM images from the list of localizations, at first an empty (black) image is created. The size of the image is determined by the lateral range the localizations span and the pixel size that is used for the rendering. The pixel size specifies the edge length of a single pixel in the resulting image.

The sampling is performed in the following way. For each localization, a two-dimensional Gaussian is assumed that is centered at the position of the localization. The value of the Gaussian distribution at the center of each pixel is assigned to the pixels. With this procedure the uncertainty of the true position for each localization is depicted. The sum of all pixel intensities that belong to a single localization adds up to the intensity of this localization.

All localizations are rendered into the same image. If a pixel is covered by multiple localizations the intensities are added. This sampling of the Gaussian distribution at the pixel centers is only performed for pixels close to the center of the Gaussian. Figure 3-10 illustrates the rendering process for a localization at the origin and a pixel size of 10 nm. The standard deviation (sigma) that defines the spread of the Gaussian can be set by the user.

![Figure 3-10 Illustration of the rendering process for a single localization. In (a) a Gaussian distribution that is centered at the origin is shown. (b) shows how the intensities for the surrounding pixels are selected: the value of the Gaussian distribution for the center of each pixel is used. The pixel size used for this example is 10 nm. In (c) only the individual pixels are visible. At a certain distance from the center of the Gaussian the intensities are almost zero and not calculated to gain performance.](image)

Pixels that are covered by many localizations get higher intensity values than pixels in regions with fewer localizations. The contrast between dense regions and less populated regions can be a problem, since the densely populated region might be so bright that the rest of the image would
appear almost black. This is, for example, the case if photostable fluorescent markers such as fluorescent beads are present during image acquisition. The intensity of regions containing these markers can easily exceed signal intensities of the structure of interest by a factor of hundred or more.

The contrast can be adjusted by defining a new maximal intensity that is much lower than the few extraordinarily high intensities present in the image. By doing so, all intensities that lie above this new maximal intensity are set to the new maximum, all other intensities retain their original value. To find this new maximum a percentile can be set. For example, a value of 0.99 defines that the new maximum is determined in such a way that 99% of the original intensities are dimmer and only one percent brighter than the new maximum.

For the rendering of 2D images, one of two different rendering modes can be chosen. One option is to render each localization in such a way that the sum of the Gaussians equals the intensity value of the localization. In this way, bright localizations are highlighted. The other option is to set the sum of the Gaussian to 1. In this case, the sum of the intensities in the final image would correspond to the number of rendered localization. That output mode can be useful to estimate the number of localizations in a certain region directly from the image. The rendered image is saved as a 16-bit grayscale Tiff image and a user defined tag can be added to the filename of the image.

### 3.2.4.1.2 Rendering of 3D images

The rendering of 3D images is in many ways similar to the rendering of 2D images. The pixel size, the spread, the percentile and the tag have the same influence on the rendered 3D image as these parameters have on the 2D image rendering.

The two differences are that the resulting image is not a grayscale but colored image and that the output mode that relates the intensities in the resulting image to the number of localizations is not available for 3D renderings.

The output of the 3D rendering module is still a two dimensional image but the information about the third dimension is indicated using different colors for different z-positions. The rendering algorithm provides a color map that assigns a color for each localization, based on its z-coordinate. With an increasing z-position, the colors change from blue to green to magenta. Figure 3-11 shows an example for a color bar used in this rendering module.

![Color bar example](image.png)

*Figure 3-11 Example of a color bar. Minimal and maximal z values are dependent on the specific set of localizations.*

### 3.2.4.1.3 Rendering of demixed images

If two channels were recorded and successfully demixed, the result can be rendered using the “Render Demixing Image” module. The pixel size, sigma, percentile and tag parameters have the same impact as for the 2D and 3D rendering. Furthermore, an option to create output with the intensities in the resulting images corresponding to the number of localizations rendered is available.

Additionally, an option is provided that determines how the demixed localizations are to be split. For each localization an angle is calculated based on the intensity ratio between the two reconstructed channels. For both colors the mean angle and the width of the angles can be
specified. Figure 3-12 illustrates both parameters. The “Render Demixing Image” module can be used multiple times with different parameters to find the optimal result. The “Render Demixing Image” module creates a 2D image with two colors, as well as a color coded 3D image for both demixed channels individually.

3.2.4.2 Text output
In addition to images text files can be stored as well. There are multiple options available. The processed localization list can be saved with the “Write Localizations to File” module to be imported again in a later session. This option can also be used to do time-consuming operations like demixing or drift correction once, store the results and import them to crop and render the data.

The second text output module is called “Write Special Output (ViSP, FRC)”. Using this module, localizations lists can be stored in the file format required for ViSP a visualization tool for SMLM data, and FIRE, a tool that measures the image resolution using Fourier ring correlation.

For both output modules, a tag can be specified that will be added to the output filename of the text file. This module also provides an option to create either output for non-demixed channels or to split the data into two channels (hereby the same parameters as used in the “Render Demixing Image” module can be applied). In the latter case, the selected output, ViSP and/or FIRE, will be saved individually for each channel.

3.2.5 Validation of the modules
In the following sections, validations for the drift correction, the connect points and the demixing module are presented.

3.2.5.1 Drift correction
3.2.5.1.1 Simulated data set
The drift correction algorithm was tested both on simulated data and real measurements of microtubules. In this section only a part of the results is shown.

The first test was performed on simulated geometrical structures as shown in Figure 3-13. A STORM data set of 15,000 frames was simulated with 100 localizations per frame and a
localization precision of 25 nm. Then a linear drift of 300 nm in x and 200 nm in y direction was applied over the 15000 frames. Subsequently, the list of localizations was processed by the feature-based drift correction. The resulting drift-free image (Figure 3-13; b) proves the functionality of the algorithm.

![Figure 3-13 Artificial test sample for the drift correction algorithm. In (a) the influence of linear sample drift on geometrical objects is shown. In (b) the result of the feature based drift correction algorithm is displayed, which removed the drift drastically. The contrast was adjusted for the left image to also show the fine lines. The scale bars correspond to 1 μm.](image)

3.2.5.1.2 Measurements
To demonstrate that the drift correction performs well not only on simulated data but also on real measurements, a 3D STORM measurement of microtubules in COS-7 cells was performed. The drift that occurred during the measurement was later corrected using the feature-based drift correction algorithm. Figure 3-14 shows the uncorrected (a) and the corrected (b) 3D STORM image and the sample drift in both lateral dimensions as well (c).

![Figure 3-14 Demonstration of the drift correction. The image in (a) shows a drift-afflicted color-coded 3D measurement of the microtubule network of COS-7 cells. In (b) the result after the feature-based drift correction is shown. The sample drift was reduced significantly. The diagram in (c) shows the sample drift determined per frame for x- and y-directions, indicating that the sample drifted towards the upper left corner. The circles indicate the center of the used chunks. The scale bars correspond to 500 nanometers.](image)

To quantify the influence of the drift correction the line profile of individual microtubule filaments was measured (Figure 3-15). Two filaments were selected that are orthogonal to each other and either parallel or orthogonal to the direction of the sample drift. The width of the line profile decreases strongly in the drift-corrected image, but only for the filament that lies orthogonal to the direction of the drift. For the other filament, no significant difference can be seen.
Figure 3-15 Quantification of the drift correction. Width of two filaments showing different orientations. The plots on the left show the sample before (a) and after drift correction (b). The line profiles of the lines in ROI 1 and ROI 2 with a width of three pixels are shown in (c) and (d). For a filament that lies orthogonal to the direction of the drift as in ROI 1 the line profile (c) of the drift corrected image shows a drastically reduced width. For filaments that are oriented along the drift the line profile (d) shows no big difference. The scale bar corresponds to 500 nanometers.

3.2.5.1.3 Influence of beads on the feature-based drift correction

To exclude a possible negative influence on the performance of the drift correction algorithm and for further validation, another measurement of microtubules was performed. The drift of the sample was in the usual range of several hundreds of nanometers per measurement. For this measurement fluorescent beads were present in the sample.

To evaluate the influence of beads for the drift correction two data sets were created from the same measurement. One data set contained all localizations, for the other data set the localizations of all beads were removed. A drift correction was performed on both data sets. The detected shifts were recorded and later used to correct all localizations, including the localizations from beads, in accordance to the detected shift. Both drift corrected images showed no detectable drift. Figure 3-16 shows the results of the drift correction for three different beads. The high spread of the individual localizations of each bead before the drift correction is in the order of hundreds of nanometers (a-c). These deviations could be successfully corrected by both the bead-aided and the purely feature-based approach (d-f). The red points were corrected using the drift estimated without the bead positions; the blue points show the result of the drift estimate using the bead positions as well. Overall, the spread of the drift correction estimated using all points is slightly lower.

On the one hand, these results show that beads improve the drift estimation. On the other hand, from other experiments it is known\[132\] that beads can also have a negative influence on measurements, e.g. by introducing artefacts to labeled structures located in close proximity of the beads. Therefore, in some cases it might be the better option not to use beads since they lead only to minor improvements in the precision of the drift correction.
3.2.5.2 Connect Points

3.2.5.2.1 Simulated data set

The performance of the “Connect Points” module was tested using a simulated SMLM data set. For the simulation, a field of view of 27 μm times 22 μm was randomly populated with 750,000 fluorophores. For each fluorophore, only one blinking event was simulated. In turn, for each blinking event, two localizations were simulated with a localization precision of 10 nm in x and y direction and 30 nm in z direction. A random frame number was assigned to the first localization. The second localization of each blinking event was assigned to the following frame. All localizations were distributed over a total of 45,000 frames in that manner.

This data set was imported to the post-processing software and processed using the “Connect Points” module. The lateral tolerance was set to 50 nm, in z-direction a 200 nm tolerance were chosen. No missing frame was allowed.

Out of 1.5 million localizations, 99.9% were connected. The same localizations were used but with randomly assigned frame numbers between 0 and 45000. In the data set with random frame numbers almost no connections should be found. The same post-processing using the same parameters was performed. This time, only 0.05% of all localizations were connected. The algorithm performs almost perfectly on the simulated data set.

3.2.5.2.2 Measurements

To validate the performance of the “Connect Points” module also on experimental data, a list of localizations from a 3D reconstruction of microtubules with 45000 frames was used. The field of view for this measurement was 27 μm times 22 μm. The reconstruction was imported into the post-processing software and the “Connect points” module was selected. The lateral tolerance
was set to 50 nm, in z-direction a tolerance of 200 nm was chosen. No missing frame was tolerated. 23% of the 1.5 million localizations were connected. A new localization list was created based on the original reconstruction with 1.5 million localizations. Position and intensity were not altered but the frame numbers were overwritten with randomized frame numbers from 1 to 45000. The same post-processing as described above was performed. In the list with randomized frames only 0.9% of all localizations were connected, demonstrating that the algorithm works as well on experimental data. The results show that the connection of points also works for experimental data. The difference between the results for simulated and experimental data will be discussed in section 4.2.

3.2.5.3 Demixing module

3.2.5.3.1 Simulated data set
To validate the demixing algorithm a test SMLM data set was simulated. The frame number, number of fluorophores per frame, the localization precision and intensity distribution were chosen in accordance to the conditions of typical demixing measurements. Random localization positions were uniformly distributed in a rectangle with 20 μm times 40 μm edge length. 40000 frames were simulated with 100 localizations per frame. Ideal demixing measurements would show the same localizations in both channels but with an individual and uncorrelated detection error. To achieve this, the localizations of both channels were Gaussian distributed around their true position with a standard deviation of 10 nm. After all localizations were simulated one of the channels was transformed using an affine transformation that introduced a shift of 100 nm in x, a shift of 50 nm in y direction and a rotation of 3 degrees. Two different fluorophores were simulated with a mean intensity of 1000 photons. The intensities were Poisson distributed. Localizations in the center of the image within a circle of a radius of 4 μm were assigned to the first fluorophore, all other localizations were assigned to the other fluorophore. A splitting of the intensities was simulated based on the characteristics of the emission spectra of Alexa Fluor 647 and CF680 and the characteristic of the dichroic mirror of the built microscope. The stochastic nature of photons were also taken into account. The localization lists for both channels were saved and imported by the post-processing software. A workflow for demixing was used to process the two localization lists. The result of this experiment is shown in Figure 3-17. The two colors were perfectly separated. In total, 96% of all localizations could be demixed.
Results

Figure 3-17 Demixing result of a test data set. Two localization lists that resemble realistic conditions during a measurement were used to create a synthetic test data set. For this test Alexa Fluor 647 and CF680 were simulated. One fluorophore is located at the center of the image within a circle of 4 µm radius. The other fluorophore is located around the circle. All localizations were randomly distributed. The demixed fluorophores are shown in red and green.

3.2.5.3.2 Measurements
To validate the demixing algorithm an experiment using Alexa Fluor 647 and CF 680 was performed. Figure 3-18 shows the result of a measurement of microtubules and mitochondria in the flat outer regions of a COS-7 cell. Despite the high demixing performance for the ideal simulated data, the demixing algorithm only finds partners for about 60% of all localizations. For reasonable borders for the color assignment this results in approximately 50% of all localizations to be assigned to one of the two colors. The reason for the difference between simulation and measurement is at least partly that some localizations are not detected by the reconstruction algorithm. If a localization is missing in one of the two channels no match can be found.

Figure 3-18 Dexmixed images of STORM measurements. The images show the microtubule network (red, Alexa Fluor 647) and mitochondria (green, CF 680) in COS-7 cells stained with primary and secondary antibodies. The scale bars correspond to 2 µm.
3.3 SuReSim

To predict the outcome of SMLM experiments, a simulation software, called SuReSim\(^{111}\) (short for Super-resolution Simulation) was developed. The purpose of SuReSim is to

- predict the results of SMLM experiments based on ground truth models
- determine sufficient experimental conditions (label, microscope, fluorophore) to successfully perform experiments and detect limitations
- test hypotheses
- create realistic SMLM data sets to validate
  - post-processing workflows
  - reconstruction algorithms
- educate beginners in the field of SMLM and help to understand the influence of different variables

The simulation software has a user-friendly graphical user interface which is shown in Figure 3-19. The result can be explored interactively and saved. The two different output modes comprise of (1) a list of localizations, similar to the output of any reconstruction software, and (2) a movie containing blinking events, similar to the raw data recorded in a SMLM experiment.

![Graphical user interface of the simulation software SuReSim. After a model structure has been imported, all parameters needed for the simulation can be specified in the “Simulation Parameters” tab and the simulated data can be interactively inspected. In this example, a model of mitochondria (red) and the simulated localizations (gray) are shown. The units are given in nm.](image)

3.3.1 Input data models (line, surfaces, binding sites)

The input for the SuReSim software, i.e. the information that defines the structure for which a STORM measurement shall be simulated, is called ground truth model. In most cases, ground truth models are based on high-resolution EM datasets, molecular structures of proteins and
Results

other structural information that has been collected about the fundamental structural organization of cells. These data provide well constrained basic models, yet some aspects of the ground truth models will have to be estimated, so these models will always contain less constrained structural assumptions. The latter can be varied and thereby different scenarios can be explored. SuReSim can help to define the range of structural possibilities that can be detected experimentally.

Each model represents a description of cellular structure using lines, triangles or preset binding sites. To model filamentous structures the WIMP file format is used. In this format individual lines are defined by separate lists of points. The points of each list are connected in the order they appear in the list and form a line.

For models that resemble surfaces, the NFF file format is used. It is a file format used by the reconstruction software suite IMOD\textsuperscript{112}, which is a widely-used software for EM reconstructions. Another option is to use the PLY format, also known as Stanford Triangle Format. For both formats only triangle-based inputs are accepted. All triangles are defined by a set of three points. The order of the points determines their orientation of the surface. For example, this is important in order to later adjust the binding angle of a label relative to the surface and to distinguish between inward and outward bound labels. The last import option is used to import binding sites for labels directly. For all other options discussed above, the binding sites for labels are determined by SuReSim based on the chosen parameters. For the last import option the binding sites are preset, a feature that is useful for simulations when the exact location of a binding site on the structure of interest is known.

### 3.3.2 Simulation parameters

SuReSim was developed to create simulations as realistically as possible. To achieve this, many different parameters can be chosen. These parameters and their influence on the simulation are explained in this section.

The parameters are grouped in different tabs, grouping parameters for the simulation (Basic settings I and II), the different output modes (Direct simulation, Tiff stack creation) and the appearance of the simulation on the screen (Visualization parameters).

#### 3.3.2.1 Basic settings I

The “Basic settings I” tab contains the most important parameters used for the simulation. Figure 3-20 shows the effect of all parameter choices in the “Basic settings I” tab on the output. The number of binding sites per nanometer of filament can be adjusted for filamentous input models. Also, the radius of the filament can be set (Figure 3-20 a, b). With this parameter, filaments of a certain diameter can be simulated and the binding sites of the labels are displaced from the center of the filament. For surface data, the number of binding sites per square nanometer can be specified. The binding sites are randomly distributed over the entire surface (Figure 3-20 c, d). If the imported model already contains information about the binding sites none of the parameters above have any effect. The labeling efficiency parameter defines the chance that a given binding site will be labeled. (Figure 3-20 e, f). Increasing the labeling efficiency has similar effects as a higher number of binding sites. A crucial attribute of SMLM experiments is the ratio between the time of the fluorophore in the on-state compared to the off-state. This ratio is set with the “On-Off Duty Cycle” parameter. It can also be interpreted as a chance for a certain fluorophore to be fluorescent in a single frame (Figure 3-20 g, h). The last parameter of the “Basic settings I” tab is the number of frames for which a SMLM experiment should be simulated (Figure 3-20 i, j). The number of localizations, especially the number of blinking events per fluorophore is increased.
both by more recorded frames and a higher blinking frequency. When bleaching is also simulated the effect of both parameters might differ. More information about bleaching is given in the next section.

<table>
<thead>
<tr>
<th>Epitopes per nm/Radius of filament</th>
<th>Binding sites per nm²</th>
<th>Labeling efficiency</th>
<th>Blinking constant</th>
<th>Number of recorded frames</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>c</td>
<td>e</td>
<td>g</td>
<td>i</td>
</tr>
<tr>
<td>b</td>
<td>d</td>
<td>f</td>
<td>h</td>
<td>j</td>
</tr>
</tbody>
</table>

Figure 3-20 Influence of the parameters of the “Basic Settings I” tab. Simulations with lower values for the tested parameters are shown in the upper panels (a, c, e, g, i), while the effects of increased values is presented in the bottom panels (b, d, f, h, j). Binding site density and radius can be altered for filamentous input (a, b). For ground truth models based on triangles the epitope density can also be altered (c, d). The influence of an increased labeling efficiency (e, f) is similar to an increased binding site number (c, d). The same holds true for the blinking constant rate (g, h) and the number of recorded frames (i, j). Both parameters influence the number of simulated localizations.

3.3.2.2 Basic settings II

More advanced settings can be found in the “Basic setting II” tab. Figure 3-21 shows the influence of most of its parameters.

A label is simulated for each labeled binding site. The angle between the model and the label can be set with the “Binding Angle” parameter (Figure 3-21 a, b). For filamentous structures, an angle of ninety degrees means that the labels are placed orthogonal to the filaments longitudinal axis. For surface data, the angle measured between label and any vector in the surface is specified. Also, negative angles can be chosen, resulting in a placement of the label within the modeled structure. Since the binding angle might not be known precisely, SuReSim offers an option to use a Gaussian distributed angle instead of a fixed one. An angular distribution can be chosen which defines the spread of the actual angle around the selected one (Figure 3-21 c, d).

For labels like antibodies a certain distance between the fluorophore and the binding site of the antibody can be set (Figure 3-21 e, f) as well as the average number of fluorophores per label (Figure 3-21 g, h).

For more realistic simulations, bleaching can be simulated by checking the “Allow Bleaching” checkbox. An exponential decrease of the number of unbleached fluorophores is assumed. The decay constant is given with the “Bleach Constant” parameter. The result can be seen by comparing Figure 3-21 (i) and Figure 3-21 (j). The effect is more pronounced when many frames are simulated.

Unspecific and uniformly distributed background labeling can also be simulated (Figure 3-21 k, l). The density per μm³ has to be set. According to this parameter, SuReSim distributes the given number of labels randomly in the available space.
During the simulation process, many random numbers are generated. If a random but fixed outcome is required, the “Reproducible Output” checkbox must be checked. All necessary random numbers are generated based on the same seed for the random number generator. This results in an identical simulation for everybody on any computer, given the same set of parameters is selected.

![Figure 3-21 Influence of the parameters of “Basic settings II” tab. The binding angle of the label can be varied freely (a, b) show an angle of 90 and -90 degree. Binding angles that vary around a given value can be simulated using the angular distribution parameter, as shown in (c, d) for no variation (c) and varying binding angles (d). The label length can also be altered (e, f), as well as the number of fluorophores per label (g, h). The effect of bleaching can be simulated (i, j). Unspecific background can also be simulated as shown in (k, l).](image)

### 3.3.2.3 Direct simulation

The direct simulation is one of two output modes. It generates a list of localizations similar to the output of a reconstruction algorithm used for SMLM data. For this workflow, additional parameters have to be chosen. The effect of two of these parameters is shown in Figure 3-22.

The lateral localization precision as well as the axial localization precision can be specified. These parameters determine the spread of the localization of repetitive blinking fluorophores. With the checkbox “Constant Localization Precision” checked, the localization precision for each localization is fixed to the specified value. Otherwise the localization precision will be coupled to simulated intensities in such a way that brighter localizations would be localized more precisely and dimmer localizations would show a higher spread. On average the simulated localization precision will match the entered value in both cases (Figure 3-22 a, b).

Reconstruction algorithms may not be able to detect each PSF. This behavior can be simulated by applying a “detection” efficiency. This parameter determines how many of all the totally simulated localizations will be shown (Figure 3-22 c, d).

After the simulation, an image can be rendered to visualize the outcome. The pixel size and the width of the Gaussian that is used for the rendering can be chosen. 3D data is rendered using a color code indicating the z values of the data. An alternative color code for 3D data which increases the contrast for red-green colorblind people can be chosen.
Parameters of the direct simulation workflow. The localization precision defines the spread of the localizations around the simulated fluorophore position (a, b). The detection efficiency determines the fraction of all simulated localizations (c) that is kept (d).

### 3.3.2.4 Tiff stack creation

The Tiff stack creation workflow creates a series of Tiff images that is similar to the raw images acquired during SMLM image acquisition.

The mean photon output defines the average number of photons simulated per blinking event. The individual intensity is determined based on an exponential intensity distribution. A minimal photon number can be specified as well. It limits the created blinking events to photon numbers that are greater than the specified minimal photon number. This parameter has to be smaller than the mean photon number. The mean blinking duration of a fluorophore defines the average time a blinking event lasts. An individual blinking event might be distributed over multiple frames, especially for large blinking durations and short exposure times. To create realistic Tiff images the pixel-to-nanometer ratio for the images has to be specified. This parameter sets the scale on the Tiff images that will be created. Another camera parameter is the frame rate. It defines the number of images taken by the camera per second. Closely related is the dead time of the camera. The dead time is a certain amount of time in which the camera does not collect any signal. It has to be lower than the exposure time per frame which is the inverse of the frame rate. To model noise, the readout noise of the analog digital converter of the camera is simulated. The readout noise is Gaussian distributed with a standard deviation of the chosen parameter. A constant offset is added as well. Both the readout noise and the offset are given in digital units. To model the behavior of EMCCD cameras, an EM gain (electron multiplying) factor can be set. The EM gain will amplify the signal by the given factor but will also add noise. With the quantum efficiency, the sensitivity of the simulated camera chip to photons can be adjusted. Only the fraction of all simulated photons given by the quantum efficiency is detected by the camera. The electrons per digital numbers conversion factor affects the intensities in the output images, higher numbers lead to smaller intensities and vice versa. The window size for the rendered PSFs of the blinking events defines to which extend the PSF is rendered. Since the PSF is less and less intense with an increasing distance its center but never reaches zero, only the inner part is rendered. To avoid artifacts of the fitting algorithm for PSFs too close to the edge of the rendered images a rim of a user-defined width can be added to the images. If a 2D PSF model is selected, parameters for the numerical aperture of the objective, the wavelength of the used fluorophore as well as the position of focus and defocus have to be chosen. All these parameters will be used by the PSF model. In case of a 3D PSF model, only a calibration file is required which contains the relation between the astigmatism caused width of the PSF in x and y direction based on the focal position (for more details see section 1.3.4.3). If the checkbox “Ensure Single PSFs” is selected the output movie will only contain isolated PSFs with no overlap. This is realized by shifting one of two PSFs that appear too close together to a new frame at the end of the movie. The “Distribute PSFs”
checkbox decides whether or not a single blinking event is split over multiple frames or if all the intensity is rendered in only one frame.

3.3.2.5 Visualization parameters
The simulated localizations can be interactively explored. The visualization parameters define the way the simulated results are displayed in the GUI. The size of points and lines can be set by using the “Point Size” and “Line Width” parameters (Figure 3-23 a, b). The line width also applies to filamentous models. The coordinate system and the related ticks can be turned on and off using the “Show Axes” and “Show Ticks” checkboxes. If the axes are turned off no ticks are displayed regardless of the status of the “Show Ticks” checkbox. The color of the coordinate system and the background can be altered, using the “Background Color” and “Color of Axes” option (Figure 3-23 c, d). For the imported model, the simulated labels and the simulated SMLM localizations a color can be assigned individually. Also, each of these three components can be hidden independently (Figure 3-23 e, f). If multiple ground truth models are imported into SuReSim the colors can be chosen for each data set individually. The rendering quality can be set between fastest and nicest. Also, the visible section of the simulated data can be cropped to smaller regions (Figure 3-22 g, h). The selected region can be kept by selecting the “Keep Borders” checkbox.

![Figure 3-23 Settings of the visualization parameter tab. Point size and line width of the individual components can be adjusted (a, b). The color of the grid and the background can be changed (c, d). The grid can also be completely turned off. The model, the label and the SMLM localizations can be turned on and off individually (e, f). To visualize only part of the data, the displayed region (g) can be cropped as in (h).](image)

3.3.3 Simulation workflow
Figure 3-24 shows an overview of the workflow of SuReSim. This section will focus on the calculations behind the simulation. The workflow begins with the import of the structure that is to be simulated (also see Figure 3-25 a). Then the parameters are chosen and the simulation is started (also see Figure 3-25 b). After that, one out of two different output modes is specified, either a list of localizations or a Tiff stack containing a movie like raw SMLM data. The key difference between both workflows is that the direct simulation is faster and the result can be explored directly, while the Tiff stack-creating workflow captures more possible imaging artifacts like the inaccuracy induced by overlapping PSFs.
Figure 3-24 Overview of the workflow for the SuReSim software. The process always starts with the import of the ground truth structures, followed by the simulation based on the chosen parameters. Two different output options can be chosen. Either a list of localizations or a Tiff stack containing PSFs similar to the movies acquired for SMLM microscopy can be created. This figure was adapted from Venkataramani et al.111.

3.3.3.1 Find binding sites
The first step after the import of a model is the determination of the binding sites. This step is skipped if binding sites are directly imported.

For filamentous data the binding sites are determined as follows. For each filament the length is calculated. Based on the length and the number of binding sites per nanometer, the number of binding sites of each filament is calculated. The radius of the filament has no influence on the number of binding sites but affects the position. All binding sites lie on an imaginary hull around the filament with the specified radius. The hull has the shape a cylindrical shape. The binding sites are equidistantly distributed along the height of the cylinder. There is one degree of freedom left: where on the circle along the surface of the cylinder at the given height the binding site is located. To find a random position on this circle for the given height, an angle between 0 and 360 degrees is chosen randomly.

As the first step in finding the binding sites for surface data the surface area for each triangle is calculated. Based on the total surface area the total number of binding sites can be calculated using the binding site density. The calculated number of binding sites is distributed over the triangles randomly but with respect to the area of the triangles (smaller triangles get fewer binding sites than larger triangles).

3.3.3.2 Find labels and fluorophore positions
Based on the labeling efficiency a subset of all binding sites is selected randomly. For these binding sites a label is determined. In general, this is done by finding a vector that rests at the location of the binding site and has a user-defined length (label-epitope distance). The direction is given by the user by choosing the binding angle. The label is simulated in such a way that the binding angle is the angle between the label direction and either the surface of the triangle or the direction of the filament. For binding angles smaller than 90 degrees again there is a degree of freedom since there are many vectors which satisfy the condition to form an angle of, for example, zero degrees. In fact, every vector that lies in the plane defined by the triangle is allowed. In these cases, one of the possible vectors is chosen randomly.

If the “Sigma of Angular Distribution” parameter is not zero, instead of the defined binding angle a different binding angle is determined by drawing from a Gaussian distribution with a mean of the chosen “binding angle” and a standard deviation of the chosen “Sigma of Angular Distribution” parameter. Then the algorithm as described above is performed. For labels containing only a
single fluorophore, the fluorophore is located at the end of the label. If multiple fluorophores are simulated for a label, the positions of these fluorophores are randomly chosen around the end of the labels, using a Gaussian distribution with a standard deviation of three nanometers.

### 3.3.3.3 Create output

Different kinds of outputs can be created. Either a list of localizations together with a rendered color-coded projection image of the simulated localizations or a Tiff stack containing simulated blinking events together with a list of locations and frames of the simulated blinking events is created.

#### 3.3.3.3.1 Simulated SMLM datasets

An illustration of the result of the direct simulation workflow is shown in Figure 3-25 (c). The simulated localizations (gray) and the rendered 3D projection (at the bottom) are shown. After the fluorophore position has been calculated, stochastic blinking is simulated. For each fluorophore, a number of blinking events is calculated based on the number of frames and the on-off-ratio. For each blinking event, the coordinates are drawn from a Gaussian distribution, centered at the location of the fluorophore, with a standard deviation in x, y and z as specified in the localization precision parameters.

If bleaching is simulated a frame for each fluorophore in which it is bleached is determined. The number of blinking events for each fluorophore is then calculated. Therefore only the frames for which the fluorophore is not yet bleached are considered. The localizations are then calculated as described above. At the end, random background is distributed in the volume occupied by the simulated localizations.

![Figure 3-25](image)

**Figure 3-25 Illustration of the direct workflow of SuReSim.** The model data in (a) is imported, binding sites and labels are simulated (b) and finally localizations originating from blinking events of the fluorophores are simulated (c). These localizations can be stored as a list of localizations or as a projection image (bottom of c). Figure adapted from Venkataramani et al.111.

#### 3.3.3.3.2 Indirect simulation workflow

Using the indirect simulation workflow, Tiff stacks, similar to raw SMLM data, can be generated. There are two different PSF models available. For 2D SMLM simulations, a PSF with a z-dependent isotropic width\(^{133}\) is used. To simulate 3D SMLM data, an elliptical Gaussian distribution with z-dependent widths along the x- and y-axis is used\(^{46}\). If the 3D-model is used the values for the z-dependent width along the x- and y-axis must be provided to the software in a table that contains the desired width at certain positions. The gaps between the given z-positions are interpolated using a cubic spline. The randomly distributed background is added. The background is considered
to consist of fluorophores that can blink multiple times. For each fluorophore, the time points of its blinking events are calculated. The photon number of each blinking event is randomly chosen from an exponential distribution with respect to the minimal photon number and the mean photon number specified in the parameter settings. For each frame of the output Tiff stack, an empty matrix with the appropriate number of fields is created, containing only zeros at the beginning. This matrix will later be saved as one Tiff image of the complete stack.

For each blinking event, the number of photons per frame is calculated based on the mean time of a blinking event and the inverse of the frame rate (exposure time). The calculated number of photons per blinking event is distributed in the fields of the matrix according to the used PSF model. Blinking events might span multiple frames.

To get from simulated photons to digital numbers (DN, the pixel values in the final Tiff stack) the photons are converted into electrons by multiplying the photon number with the quantum efficiency. All entries of the matrix are multiplied with the EM gain factor. Each field of the matrix is replaced with a value drawn from a Poisson distribution with the mean and the standard deviation of the original value of the field in the matrix. This simulates the noise introduced by the EM gain. All fields are divided by the electron per DN parameter. After that, the read-out noise is added by sampling from a Gaussian distribution centered at the constant offset value with a standard deviation specified in the parameters.

3.3.4 Additional Features

3.3.4.1 Editor

SuReSim also includes an editor which can be used to create 2D models of filaments based on imported images. Figure 3-26 shows the GUI of the editor. Filaments can be drawn by simply clicking on the location in the imported image where a point of the filament is positioned. The image can also be zoomed in for more precision. After the pixel-to-nanometer ratio of the imported image has been set and some lines have been drawn, these lines can be exported in the WIMP file format.

Using the editor is a fast way to create models of filamentous structures, for example to validate results from measurements.
3.3.4.2 Tool for the validation of reconstruction algorithms

SuReSim includes an option to create realistic Tiff stacks and ground truth data that contains the true position of the fluorophores that were simulated. Both can be used to validate the performance of reconstruction algorithms for SMLM data. The simulated Tiff stack can be reconstructed by a reconstruction algorithm. Subsequently, the output of the reconstruction algorithm can be compared to the true positions of the fluorophore by using the “Evaluate Reconstructions” feature of SuReSim. SuReSim tries to find matches between localizations in the reconstruction and the ground truth. A match is defined as a localization that appears in the same frame close to a position in the ground truth data. The tolerance for these matches can be altered.

The validation tool searches for matches and detects the number of true positive localizations in the reconstruction. A localization is considered to be a true-positive if it is close enough to a point in the ground truth data. Also false-positive localizations, localizations that appear in the reconstruction but have no matching point within the ground truth data set, are calculated. The number of false-negative localizations describes localizations that are missing in the reconstruction but appear in the ground truth.

To compare two data sets one of the data sets can be shifted relatively to the other. This compensates for the fact that different reconstruction algorithms use different coordinate systems, where either the center of a pixel or its edge is the origin of the coordinate system. Many quantities like precision, Jaccard-index or f-score as well as information about the root mean square error between the localizations and the ground truth are calculated.
Results

Figure 3-27 GUI for the validation of reconstruction algorithms implemented in SuReSim. On the left, the interface of the validation tool for reconstruction algorithms is shown. It is used to compare the output of a reconstruction algorithm on a simulated Tiff stack with the known true positions. On the right, different performances with increasingly relaxed tolerances for the matching of ground truth and reconstruction result are shown. Figure adapted from Venkataramani et al.111.

3.3.5 Qualitative results on test systems

3.3.5.1 Microtubules

For a qualitative comparison between measurements and simulated data microtubules were chosen as the first test system. Microtubules form long filaments of 22 nm diameter with 1.625 binding sites per nm. For this comparison, a STORM image of microtubules stained with a primary antibody against alpha tubulin and a secondary antibody labeled with Alexa Fluor 647 was recorded. This image was used to draw similar filaments using the editor of SuReSim. The model was imported and a simulation was performed with the same parameters that were used during the image acquisition of the STORM image. Unspecific binding was simulated with values estimated by the number of unspecifically bound labels in the background of the measurement. The localization precision was estimated using the approach of Endersfelder et al. 55. The labeling efficiency was estimated to be 10%. Figure 3-28 shows the resulting images for the simulation and the measurement. Besides the good agreement in the general structure of the filament, the inhomogeneity in the density of localizations is well captured by the simulation. Furthermore, the increased intensity of the projected image at the rim of the microtubules and the reduced intensity in its center are visible in both images.

The main difference between simulation and measurement are parts where the filaments in the measurement appear to be broken or discontinuous. The reasons for that might be that these parts of the microtubules are blocked so that the antibody cannot reach them or that the structures in these regions could be physically broken. This was both not considered when creating the ground truth model. In conclusion, the simulation adequately captures the features detected in the experiment.
Results

Figure 3-28 Comparison between simulation and experiment for microtubules. (a) shows the simulated and (b) the measured microtubules, stained with antibodies against α-tubulin. The scale bar corresponds to 500 nm.

3.3.5.2 Mitochondria

Mitochondria were chosen as the second test system. They form round structures of a diameter of up to several micrometers. The outer membrane can be labeled using primary antibodies against TOM20, an outer membrane protein distributed over the entire surface and secondary antibodies.

For the comparison an electron tomography model of mitochondria was used\textsuperscript{119}. Parameters for the simulation were taken from the measurement and from literature\textsuperscript{120}. A qualitative comparison is shown in Figure 3-29. A z slice of 200 nm thickness was projected onto one plane. This results in a more dense signal at the edge of the mitochondria. Both images look very similar, the most obvious difference arises from the fact that the mitochondria in the model and in the measurement differ in size. However the pronounced edges, the labeling inside this edges and the unspecifically bound background look very similar, suggesting that the simulation captured the relevant parameters and therefore produces a realistic result.

Figure 3-29 Comparison between a simulated z slice of mitochondria and the measurement. (a) shows simulation and (b) an antibody staining against TOM20, a marker for mitochondria. The scale bars corresponds to 500 nm.
3.3.5.3 Nuclear pore complex

As the third test system, the nuclear pore complex was chosen. It is a highly symmetrical structure that is situated in the membrane of the nucleus. Certain proteins like nucleoporin 133 (nup133) show an eightßfold symmetry in the projection, but have 4 stacked copies of the protein lying on top of each other in each of the nodes of the octagon.

The model for an individual nuclear pore was created by placing eight binding sites on an octagon with an edge length of about 40 nm and creating four copies of the binding sites to simulate the stacked proteins. This model is only used for 2D projections and simulations. A larger model was then created by placing a number of individual pores at random positions on a plane.

The model, the simulation and the measurement of nuclear pores with SNAP-tag labeled nup133 protein is shown in Figure 3-30. The parameters for the simulation were chosen based on the measurement, a labeling efficiency of 43% was assumed for the SNAP-tag label. The comparison between simulation and measurement show the strong similarity between both images, indicating a realistic simulation.

![Figure 3-30](image)

Figure 3-30 Comparison between simulated nuclear pore localizations and measurements. (a) shows the model used to simulate the nuclear pore complex shown in (b). (c) shows a comparison with measurements of SNAP-tagged nup133, recorded by Anika Rauf (Heilemann laboratory, Frankfurt Main, Germany). Scale bar corresponds to 100 nm.

3.3.6 Quantitative evaluation of SuReSim

The qualitative comparison between measurements and simulation are satisfactory, but not sufficient, to proof that SuReSim does create realistic simulations. To also quantitatively validate the simulations, two different measures were used. Two published method to estimate the resolution of SMLM images called FIRE and the nearest neighbor in adjacent frames (NeNA) method were used.

3.3.6.1 Evaluation using the FIRE software

The principle behind the FIRE algorithms is that a set of localizations is split randomly in two halves. For both sets, a 2D projection is rendered and then transformed into Fourier space. The Fourier transformed signal is then correlated along circles of constant spatial frequency. The spatial frequency where the correlation drops under the threshold of 1/7, can be translated into a resolution present in the image.

For this evaluation, the model shown in Figure 3-31 (a) was used. Labeling efficiencies of 10, 50 and 100 percent were used as well as localization precisions of 25, 12, 8 and 4 nm. A Tiff stack was simulated and reconstructed using RapidSTORM for each combination of these parameters resulting in 12 simulations shown in Figure 3-31. Table 3-2 shows the results of the Fourier ring correlation analysis performed for each simulation.
As expected, simulations of better imaging conditions lead to better values for the calculated resolutions, indicating a realistic simulation. The fact that the resolutions of the FIRE algorithm and the localization precision does not match in absolute terms was also anticipated since the resolutions that are reported by the FIRE analysis are always higher than the localization precision. Even for a localization precision of 0 the FIRE resolution will still be finite (see supplementary information from Nieuwenhuizen et al.\textsuperscript{56}).

![Simulation Parameters](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitope density</td>
<td>1.625 nm(^{-1})</td>
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<tr>
<td>On-off duty cycle</td>
<td>1/2000</td>
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<tr>
<td>Radius of filaments</td>
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<tr>
<td>Direct simulation</td>
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<tr>
<td>Label epitope distance</td>
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<td>Bleaching</td>
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<td>Fluorophores per label</td>
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<td>Background label</td>
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<tr>
<td>Labeling efficiency</td>
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<tr>
<td>Localization precision</td>
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<tr>
<td>Recorded frames</td>
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<tr>
<td>Binding angle</td>
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</table>

Figure 3-31 Quantitative evaluation based on FIRE. Model of two microtubule strands (a) and simulations for different parameters (b-m). The localization precision increases from left to right, the labeling efficiency increases from top to bottom. The scale bar in (a) corresponds to 100 nm and applies to all images. Figure adapted from Venkataramani et al.\textsuperscript{111}. 
Table 3-2 Fourier ring correlation calculation of the resolutions. Results for simulations of two microtubule strands with different parameters. As expected, the calculated resolutions get better with increasing labeling efficiency as well as with a better localization precision. The units for the calculated FIRE values are given in nanometers. Table from Venkataramani et al.\textsuperscript{111}.

<table>
<thead>
<tr>
<th>Labeling efficiency</th>
<th>Localization Precision</th>
</tr>
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<tr>
<td></td>
<td>25 nm</td>
</tr>
<tr>
<td>10%</td>
<td>110.9 ± 0.66</td>
</tr>
<tr>
<td>50%</td>
<td>96.4 ± 0.43</td>
</tr>
<tr>
<td>100%</td>
<td>91.5 ± 0.29</td>
</tr>
</tbody>
</table>

3.3.6.2 Evaluation simulating different experimental conditions

For a second quantitative validation of SuReSim, different experimental conditions were simulated. The influence of the number of photons from individual blinking events, which is for high frame rates proportional to the exposure time, was evaluated. Experimental data of the microtubule network was recorded with different exposure times. The longer the exposure time, the more photons are collected per blinking event per frame, resulting in better localization precision. The low localization precision leads to blurry structures. For this experiment STORM movies were recorded using three different exposure times of 4, 8 and 16 ms. All movies were taken from the same spot of a sample of microtubules. Only short movies were recorded to reduce photobleaching. After the STORM movies were reconstructed, 2D models were created using the editor of SuReSim. The mean number of photons per PSF per frame was determined for each exposure time. Subsequently, Tiff stacks were simulated based on the model derived from the measurement. Parameters like the binding site density were taken from literature\textsuperscript{113}. The mean photon number was set to 1300, 1800 and 2700 photons. These Tiff stacks were reconstructed using RapidSTORM. For two regions of interest with different filament densities the simulation and measurements are shown in Figure 3-32 together with an overview over the whole recorded field of view. Both measurement and simulation show similar results. For both ROIs the image quality increases with higher photon numbers.

The localization precision for the whole field of view was determined for each exposure time using the nearest neighbor in adjacent frames (NeNA) approach\textsuperscript{55}. In the same manner the localization precision was calculated for all three simulations. After that the Fourier ring correlation values were calculated, both for the whole field of view for simulation and measurement. Table 3-3 summarizes the results. The localization precisions between simulation and measurement match very well. Also the expected behavior of increased localization precisions for a higher photon number is seen both in the experimental data and the simulations. The resolutions calculated using the Fourier ring correlation show also the expected trend of smaller and therefore better resolutions at higher photon numbers. The comparison between measurement and simulation with respect to the FRC resolutions shows not identical but very similar values. Besides the similar qualitative comparison also the quantitative measure shows the same trend with similar values showing a deviation of less than 10%. This comparison shows the realistic Tiff stacks that can be simulated using SuReSim.
Table 3-3 Comparison between the localization precision and Fourier ring correlation resolution between measurements and simulations. For the measurements microtubules were imaged with different exposure times. The mean photon number per blinking event was determined for each measurement and used for the simulations. The localization precision was determined for both the experimental data and the simulated data using the nearest neighbor approach. Table from Venkataramani et al.

<table>
<thead>
<tr>
<th></th>
<th>Experiment</th>
<th>Simulation (Tiff stacks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure time/photons</td>
<td>4 ms/1320</td>
<td>8 ms/1802 16 ms/2709</td>
</tr>
<tr>
<td>Localization precision</td>
<td>19 nm</td>
<td>15 nm 12 nm</td>
</tr>
<tr>
<td>FRC Resolution</td>
<td>97.4 ± 1.03 nm</td>
<td>86.9 ± 0.79 nm 79.9 ± 1.03 nm</td>
</tr>
<tr>
<td>FRC Resolution</td>
<td>102.4 ± 0.74 nm</td>
<td>88.8 ± 0.29 nm 74.1 ± 1.44 nm</td>
</tr>
</tbody>
</table>
Figure 3-32 SMLM images and simulations for different photon numbers of the PSFs per frame. A sample of microtubules (a) was recorded with varying exposure times (b-d) and (h-j). Models were created based on the SMLM image of the microtubule filaments of both ROIs. The mean photon number per PSF per frame was determined for each exposure time and used together with the model of the structure to create Tiff stacks. (e-g) and (k-m) show the results of the reconstruction of these Tiff stacks. Measurement and simulation show that the image quality degrades with lower photon numbers captured. The scale bar in (a) corresponds to 1 μm. The scale bar in (b) corresponds to 200 nm and applies for (b-m). Figure adapted from Venkataramani et al.111.
3.3.7 Contributions of others to SuReSim

Not all of the work on SuReSim was done all by myself.

The general idea for the simulation originated from a discussion of me and Varun Venkataramani. The programming and developing of the algorithms was done by me, except marked otherwise. The sample preparation and the acquisition of EM images were performed by Varun Venkataramani. Part of the GUI for SuReSim was designed and programmed by Maximilian Scheurer based on a Matlab version of the software written by myself. Part of the algorithms for the creation of Tiff stacks were written by Niels Schlusser under my supervision. The figures used from the publication of SuReSim\textsuperscript{111}, were created jointly as it is reflected by the shared first authorship.
3.4 Molecular architecture of presynaptic nerve terminals

After finalizing the microscope as well as the control and the post-processing software, experiments on tissue sections of the auditory brain stem containing the calyx of Held giant presynaptic terminal were performed. Subsequently, the STORM recordings were validated using SuReSim software.

3.4.1 STORM measurements of F-actin and the synaptic vesicle cluster

To investigate the possible role of F-actin at the SV cluster and the AZ, dual color STORM measurements of F-actin, labeled with phalloidin and antibody-labeled synaptophysin, were performed on 600 nm thick cryo-sections. Sample preparation was performed by Varun Venkataramani. For imaging of the vesicle clusters, regions showing the calyx of Held were chosen. In Figure 3-33 the F-actin signal forms a large (several tens of micrometers) ring structure (red), indicating the shape of the calyx of Held and framing the synaptophysin-positive (green) presynaptic area.

![Figure 3-33 STORM measurement of a calyx of Held of a rat. F-actin (red) and synaptophysin (green) were labeled. The scale bar corresponds to 5 μm. Experiment performed together with Varun Venkataramani.](image)

To further assess a possible role of F-actin for the SV cluster, magnified STORM images of synaptophysin (green) and F-actin (red) were analyzed (i.e. Figure 3-34). Actin does surround the SV clusters. Interestingly, the F-actin signal also overlaps with the synaptophysin signal, suggesting that actin might be indeed involved in SV clustering. However, even with the new STORM microscope providing a localization precision of about 10 nm, the actin distribution in the SV
cluster shows no filamentous structure and appears to be dotty and discontinuous. Neither small, SV connecting filaments nor long isolated actin strands could be resolved. Furthermore, individual SVs could not reliably be identified. To explain the experimental results, simulations of F-actin and SVs were performed and analyzed. The results are shown in the following section.

Figure 3-34 STORM measurement of the calyx of Held from a rat brain. On the left (a), an overlay of the synaptophysin signal (green) and actin signal (red) is shown. There is actin signal outside as well as within the synaptic vesicle cluster. In the middle panel (b), the synaptophysin channel is shown, the image on the right panel (c) shows the actin channel, the scale bar corresponds to 500 nm and corresponds to all images.

3.4.2 SuReSim simulations of F-actin and synaptic vesicles at the active zone

This section presents the results of the simulations of actin and synaptophysin at the SV cluster.

3.4.2.1 Simulation for F-actin

As the experimental results obtained using STORM could not uncover a clear structural interrelation between actin and SVs, the limits of SMLM were validated by means of the simulation software, SuReSim. Figure 3-35 shows the ground truth model (a), described in section 2.5.1, and the result (b) of the simulation of F-actin (see section 2.5.1 for parameters), labeled with phalloidin in the vesicle cluster. The ground truth shows distinct small filaments of a length of only a couple of tens of nanometers, whereas the simulation shows no filaments but clustered localizations.

For the simulation in Figure 3-36 an antibody staining was simulated to show the label density more pronounced. In Figure 3-36 (b) the structure, the labels and the simulated SMLM localizations are displayed in one picture. It is hard to assign individual localizations to filaments, even with the filaments and the labels shown in the same picture.

If only the SMLM localizations are considered as shown in Figure 3-36 c) it is impossible to identify individual filaments. This also holds for the 2D projection of the simulated data as shown in Figure 3-35 (b).

Figure 3-35 Simulation of F-actin within a SV-rich region. (a) shows the actin model which was derived from EM reconstructions. (b) shows the projection of the simulated outcome of a STORM experiment. The color bar ranges from 0 nm to 380 nm. The contrast in (b) was increased, the scale bar corresponds to 200 nm and applies to both images.
Figure 3-36 SuReSim simulation for antibody labeled actin. In (a) the actin model (red lines) and the simulated labels (yellow lines) can be seen. (b) shows additionally the simulated localizations (gray dots). (c) shows the simulated localizations only. Units are given in nanometers. Simulation performed by Varun Venkataramani.
While these simulations explain the experimental observations, the question remains if an even better STORM microscope could discriminate the short actin filaments within the presynaptic vesicle cluster. Such simulations for better cases are shown in Figure 3-37. A realistic lateral localization precision of 4 nm and 8 nm axial were simulated and the results shown in Figure 3-37 (b). An unrealistically good localization precision of 1 nm both lateral and axial was simulated in Figure 3-37 c). Figure 3-37 d) shows a simulation for an additionally high labeling efficiency of 100%.

Figure 3-37 SuReSim simulation for phalloidin labeling. (a) shows the F-actin model, (b) simulation using the best publish localization precision so far. A unrealistically high localization precision of 1 nm both lateral and axial was simulated in (c). In (d) additionally an unrealistically high labeling efficiency of 100% was simulated. The scale bar corresponds to 200 nm the color bar to 200 nm.

From these experiments and the simulation can be concluded that short filaments placed at high density in an unordered spatial arrangement are close to impossible to discriminate, even with the highest resolutions and with a labeling efficiency of 100 percent; dense region are not resolved. Noteworthy, the direct simulation workflow was used, thus these simulations represent best case scenarios without any distortions originating from overlapping PSFs, imperfect switching behavior or unspecific bound background localizations.
3.4.2.2 Simulation for synaptic vesicles

Simulations for synaptic vesicles (the used model and parameters are described in section 2.5.2) were performed based on the known average copy number of synaptophysin in synaptic vesicles and an assumed labeling efficiency of 10%. A localization precision of 10 nm in lateral dimension and 30 nm in axial dimension was used to simulate the resolving power of the custom built microscope. Figure 3-38 and Figure 3-39 show the results of these simulations. In Figure 3-39 (a) the vesicle model (red spheres) and the simulated labels (yellow lines) are displayed. On average 3 labels are simulated per vesicle. The overlay of the model (red spheres), the labels (yellow lines) and the simulated SMLM localizations (gray dots) are shown in Figure 3-39 (b).

When only the localizations are visible as shown in Figure 3-39 (c), it becomes almost impossible to detect individual vesicles. Also the projected image as in Figure 3-38 (b) shows no clearly distinguishable vesicles at dense regions. Remotely lying vesicles might be differentiable but in regions with a high vesicle density the localizations of individual vesicles overlap and form a continuous cloud. Hence, individual SV cannot be discriminated within densely packed regions of the SV cluster due to the limited resolution of the microscope and labeling parameters derived for synaptophysin. Again the direct simulation workflow was chosen, which means that Figure 3-38 and Figure 3-39 show the best case scenario.

Figure 3-38 Simulation of synaptic vesicles. (a) shows a projection of the model used for the simulation of synaptic vesicles. (b) shows the projection of the simulated result. No individual vesicles can be identified within densely packed regions of the SV cluster, yet in its periphery, single SV might be visible. The contrast was enhanced in (b), the scale bar corresponds to 200 nm, the color bar to 430 nm.
Figure 3-39 SuReSim simulation for synaptophysin at synaptic vesicles. In (a) the vesicle model (red spheres) and the simulated labels (yellow lines) can be seen. (b) shows additionally the simulated localizations (gray dots). (c) shows the simulated localizations only. Units are given in nanometers.
3.5 Limits of SMLM assessed using SuReSim

In the last section, limits of SMLM measurements for actin filaments and synaptic vesicles were discussed. This section will show examples of simulations that further illustrate the limits of SMLM techniques.

3.5.1 Limitations introduced by labeling efficiency, localization precision and packing density

To exemplify the effect of labeling efficiency and localization precision, the actin distribution in erythrocytes was chosen, because EM ground truth data was available. Several simulations of actin in erythrocytes were performed with varying localization precisions and labeling efficiencies (detail about the model and the parameters used can be found in section 2.5.5). The results are shown in Figure 3-40.

For dense actin networks as in the erythrocyte both the labeling efficiency and the localization precision are equally limiting factors. The model contains filaments with a length between 5 and 50 nm. The filamentous structure of actin is not visible even when all binding sites are labeled and the image is acquired with the best setup available, as can be seen in Figure 3-40 (m). Noteworthy, the direct simulation workflow and therefore the best case scenario was simulated.

Figure 3-40 Example of the influence of labeling efficiency and localization precision. A phalloidin labeling was simulated for the model (a) of subcortical actin at the membrane of erythrocytes. The simulated localization precision increased from left to right, the labeling efficiency increases from top to bottom. Even with highest labeling efficiencies and the best localization precision currently possible no filaments can be resolved. Scale bars correspond to 100 nm. Figure was taken from Venkataramani111 and modified.

3.5.2 Limitations introduced by the label epitope distance and label orientation

In another example, simulations of the same SV model and parameters as in section 3.4.2.2 were used to simulate different configurations of the label. The simulations were performed for outward binding labels with a label epitope distance of 16 nm, for labels that are within the membrane (label epitope distance equals zero) as well as for inwardly bound labels. For this simulations, the best localization precision currently achievable of 4nm in x and y direction and 8 nm in z direction was used46 (Figure 3-41).
In case of the outward bound label, individual vesicles cannot be discriminated in densely populated regions of the cluster. In case of the membrane associated labels (i.e. 0 nm distance between fluorophore and epitope), high labeling efficiencies help to distinguish individual vesicles; yet in regions of high density a quantitative result is still not achievable. For the inward bound case, individual vesicles are clearly distinguishable. Even in dense regions individual vesicles can be discerned by additionally considering their z position (not shown in the figure). Hence, this example shows that SuReSim can be successfully used to develop new experimental strategies that promise a tractable experimental solution.

![Simulation of different binding angles and labeling efficiencies for a SV cluster.](image)

**Figure 3-41** Simulations of different binding angles and labeling efficiencies for a SV cluster. Different sets of parameters were simulated for the ground truth model (a). From top to bottom the labeling efficiency is increased. In the left column (b-d) antibody staining with primary and secondary antibodies is simulated. The label is assumed to bind outwards. In the middle column (e-g) a membrane associated label is simulated. The column on the right (h-j) shows a simulation for an inward bound label. Individual vesicles can be determined for the membrane associated label but even better for the inward bound label. Figure taken from Venkataramani et al. The scale bar corresponds to 100 nm.

### 3.5.3 Misinterpretations of STORM images

Interpretation of SMLM data might be difficult due to the nature of the reconstructions often showing a point cloud appearance. For the simulation in Figure 3-42, the same ground truth was used as in section 3.5.1. From the simulation (Figure 3-42 b), in turn, a new ground truth model was derived which is shown in Figure 3-42 (c). When performing SMLM experiments only information as shown in Figure 3-42 (b) is available and can easily be misinterpreted.

A simulation with the same parameters performed on the deduced model of five actin filaments forming an actin bundle, resulted in an image (Figure 3-42 d) very similar to the initial simulation based on the ground truth model. Thus, one has to be very careful with the interpretation of experimental results. Fine structures will not be resolved and might lead to oversimplified assumptions.
Artifacts in SMLM. In (a) a fine structure of subcortical actin from an erythrocyte is shown. (b) shows the simulation based on (a). Next, from the rendered image the easiest model of an actin bundle was deduced and is depicted in (c). The result of the simulation of (c) with the same parameters as for (a) is shown in (d). Despite the different ground truth models the SMLM images look similar. The scale bar corresponds to 100 nm. Figure adapted from Venkataramani\textsuperscript{111}.

The effect of labeling efficiency is further illustrated using the SV cluster already introduced before (Figure 3-43). For realistically low labeling efficiencies, the simulation resulted in small clusters showing the size of SVs. These clusters originate from individual fluorophores that blink multiple times but can be easily confused with whole SVs. Background staining that cannot be completely avoided in experiments would make it even harder to distinguish between sparse SV signal and unspecific labeling. Thus, SuReSim proves to be a good tool to prevent overinterpretation by comparing the results of ground truth structures with experimental results.

Figure 3-43 Example for the influence of labeling efficiency. The model (a) is used for simulations of synaptophysin-labeled SVs with increasing labeling efficiency (b-d). Especially for low labeling efficiencies some single clusters can be seen that can easily be confused with synaptic vesicles. The scale bar corresponds to 100 nm. Figure modified from Venkataramani\textsuperscript{111}. 
4 Discussion

4.1 STORM setup and microscope control software

The STORM setup implemented in this thesis combined recently established designs and concepts\textsuperscript{1,66,110}, yet goes beyond a mere recombination of existing modules. It has proven to achieve excellent resolution in 3D, drift stability and dualcolor imaging free of chromatic aberration artefacts. The combination of this hardware design with user-friendly interfaces controlling the microscope as well as the integrated postprocessing workflow resulted in a robust system uniquely suited for 3D dualcolor STORM imaging experiments. At the time of its conception, comparable systems did not exist, even today comparable solutions only exist in the form of custom built systems in expert laboratories, yet commercial sources are not available. The desired performance of the custom-built microscope was confirmed with a set of validation experiments. The pixel size deviates only by less than one percent from the theoretical value (see section 3.1.2.1). Both channels are well-aligned and show no significant distortions. Since only one channel is used to determine the position of the localizations, minor distortions can easily be compensated by the demixing algorithm of the post-processing software. The achieved localization precisions of 6.5 nm for 2D and 9.4 nm for 3D match the theoretical values calculated with the method by Thompson\textsuperscript{53} (5.8 nm for 2D and 7.5 nm for 3D) or Mortenson (6.8 nm for 2D and 8.6 nm for 3D).

The active drift stabilization improves the stability of the focal position significantly. Small inaccuracies in the range of ten to twenty nanometers have to be taken into account. These small focal shifts are below the range of the achievable z resolution and can be further reduced by post-processing steps. These small focal shifts are comparable to other focus stabilization systems where shifts in the range of 20 nm are reported\textsuperscript{134}. The spectral demixing approach prevents chromatic aberration which could otherwise cause several tens of nanometers\textsuperscript{25} of distortion between both channels for dual color imaging.

The microscope control software performs reliable and provides easy access to most features of the microscope. Not all components are implemented into the software yet. The implementation of the control of the two additional lasers (488 nm and 561 nm wavelength) and the motorized stage moving the sample in x and y direction is currently missing. However, this does not limit the functionality of the microscope as these elements can be accessed by external software. The microscope control software automatically generates a specific folder hierarchy enforcing a structured and constant organization of data. This standardized way of storing data ensures unique filenames for parent folders and output files enabling an unequivocal assignment of data.

Furthermore, the custom-made software also provides a possibility to start the reconstruction of the recorded images already during the measurement with only a small delay. The workaround using Python ensures the flexibility to implement different reconstruction algorithms or to automatically start the post-processing software in future releases. In summary, a robust software for the acquisition and pre-processing of STORM data was developed and validated.

4.2 Post-processing software

The decision to write a custom-made post-processing software was motivated by the fact that there is currently no free software package available that provides all features needed for post-processing of SMLM data. The post-processing software combines an easy-to-use and comfortable GUI and high flexibility of a complete post-processing workflow. The performance is dependent on the size of the processed data and the selected parameters, but programming in
Java results in fast execution of the individual steps. Writing individual modules in the C or C++ programming language might have further increased the performance, however would have made the programming more challenging and the distribution of the software to other operating systems like Linux or Mac OS more cumbersome.

Quality-wise, the performance was tested both on simulated and measured data sets. The drift correction performs well as long as the necessary conditions, like a sufficient localization number per frame, are present in the data. The demixing algorithm performs almost perfectly on simulated data sets and sufficiently well on real measurements. For measurements one of the two colors can be assigned to approximately 50% of all localizations, a higher efficiency than the 25% reported by Lampe et al\(^1\). Partly, this might be caused by more conservative thresholds. The bottleneck for the demixing is the proper fitting of the PSFs from the raw data. If a localization is missing in either channel no partner can be assigned. Reasons for missing partners can be missed detections by the reconstruction software (especially for low photon numbers) or overlapping PSFs that influence one channel stronger than the other and lead to displaced reconstruction results. There might also be small non-linear distortions between the two channels that cannot be corrected by an affine transformation.

The number of connected localizations for the experimental data set (23%) is lower than expected, especially when compared to the simulated data set (99.9%). The exposure time for this measurement was chosen to match the mean blinking duration of the fluorophore. The expectation would be that almost every blinking event is distributed over two frames. The low number of actually connected points indicates that many blinking events that are distributed over multiple frames are either not fitted or fitted but sorted out because of a too low photon number and therefore not connected. Additionally no effects like overlapping PSF that can distort the reconstruction were present in the simulated data set. The number connections found in the randomized experimental data set was about 1%. It is high compared to the results for the simulated randomized data set. The reason for the higher number of connections in the experimental data set is the higher fluorophore density. All localizations originate from the area covered by microtubules, which is only a fraction of the complete field of view. This increases the chance that two localizations from different fluorophores that are close together are connected, even when random frame numbers were assigned.

The microscope control software together with the automated reconstruction forms a unit with the post-processing software, enabling an efficient and convenient workflow for STORM experiments. To improve the reliability and documentation of the acquired data, all output files are located in a folder that is created in the directory of the imported localization list.

### 4.3 SuReSim

SuReSim gives the opportunity to simulate the results of SMLM imaging for arbitrary 3D structures. While SuReSim covers almost all relevant parameters that determine the outcome of an experiment, there are a few issues that are difficult to consider in the simulation process. For example, the effect of molecular crowding and occlusion of binding sites by other structures is difficult to incorporate quantitatively. Occlusion can be partly considered by reducing the labeling efficiency, but the labels are still randomly distributed over the structure with no respect to binding sites that might be occluded by the ground truth structure. Another potential restriction is that despite the fact that many parameters can be set for the simulations, not all of these parameters may be known with sufficient precision. For example, the epitope density for certain
proteins or the blinking characteristics for the used fluorophore might be unknown. However, the number of publications that quantify fluorophore properties and protein compositions is steadily increasing. If there is no data available, an educated guess may help to obtain first insights. Other parameters like the number of unspecific background can be determined from SMLM measurements.

The model creation is the most cumbersome part of the simulation workflow. Either EM data has to be contoured or structures have to be simulated based on assumptions or prior knowledge. A large number of EM models have been published that could be of potential interest for localization microscopy measurements. Though the structure could be potentially revealed by SMLM, one often is interested in the nanoarchitecture of protein organization of many proteins, e.g. the calcium channel organization in the active zone and its surrounding environment. Clusters have been described but whether they can be resolved with SMLM is unclear. EM models have described presynaptic structures such as connectors and tethers but whether they can be resolved by SMLM is unclear as well.

The simulation of the labels places all simulated fluorophores at the end of the label. This is an assumption made for the sake of simplicity. The influence of this simplification depends on other parameters. If a single objective microscope is simulated the localization precision plays a larger role than the imperfect fluorophore position. For small labels carrying only one fluorophore per label, the effect is also negligible.

For the 3D Tiff stack simulation workflow only a relatively simple PSF model is available. Despite its simplicity the most common reconstruction artifacts, such as overlapping PSFs, can be simulated. Future versions of SuReSim could include the option to import custom PSFs for the Tiff stack simulation. Users could measure the PSF for their microscope and get even more realistic and customized predictions whether or not a given research question can be addressed with the given microscope. Also simulations for other 3D techniques like the double helix approach could be realized that way.

The quantitative as well as the qualitative evaluations show that the simulations are in good agreement with the measurements despite the above mentioned inaccuracies. Nevertheless, SuReSim simulations should be considered as a best case scenario. Real measurements might introduce additional quality decreasing factors such as imperfect buffer conditions or inhomogeneous laser profiles.

A striking advantage of SuReSim compared to other simulation software packages is that arbitrary 3D structures can be imported and labels can be simulated realistically with many relevant parameters. This is of special importance if densely packed structures are simulated where the label length has the same order of magnitude as the distances between individual structures. SuReSim can be used in different ways. The simulations can be used to evaluate whether SMLM is capable of resolving the structure of interest. Simulations can be performed using best, realistic and worst case parameters. SuReSim can be a profound tool for the planning of experiments. Existing workflows for reconstruction and post-processing can be evaluated using SuReSim. This way analysis pipelines can be tested even before experimental results are acquired, helping to plan and speed up projects.

Another use is the testing of hypothesis. Experimental results might be used to conclude models of new structures. These models can be imported to SuReSim and evaluated. By simulating the same structure multiple times, it can be distinguished between artifacts based on the stochastic labeling or true structures that can be seen in any simulation. This might help to prevent the over-interpretation of experimental results.
SuReSim can also be used to teach the importance of certain parameters, such as the label length, for SMLM. Different simulations can be performed and interactively compared. In that sense SuReSim might be the perfect tool for beginners in the field of SMLM. Creating Tiff stacks teaches the indirect image formation process of SMLM, different simulations with varying photon numbers might directly teach the influence of different fluorophores. Since all simulations can be performed on the very same structure, the influence of the chosen parameters are more pronounced than by comparing experimental results.

4.4 3D visualization of F-actin and synaptic vesicles
The results of the simulation for F-actin labeled by phalloidin and antibodies are shown in Figure 3-35 and Figure 3-36. The direct simulation of actin-attached antibodies indicates one major problem – a low number of labels per filament. This is partially due to the fact that presynaptic actin filaments are so short that they only contain 10 to 15 binding sites. With a low labeling efficiency of ten percent on average only one or two binding sites per filament are occupied by a label (compare Figure 3-36; a), but also completely unlabeled filaments may exist. Not only the low number of labels but also the high spread of the localizations from a single fluorophore makes it hard to see the underlying filaments. Especially in dense areas where many filaments are close together only a cloud of points is visible. Another important point to notice is that the structures visible in SMLM measurements might be over-interpreted. The image shown in Figure 3-35 (b) might lead to the impression that there are clusters of actin or some thicker filaments, while there are only many densely packed filaments which are not perfectly labeled. The actin model was derived from electron microscopy data by selecting filaments based on their diameter of up to 10 nm. Therefore, it is possible that intermediate filaments, that show a diameter of approximately 10 nm were false-positively identified as actin. However, that does not affect the general conclusions made here.
Also the simulation of synaptophysin at synaptic vesicles shows similar results. Although SVs have a relatively large diameter compared to the resolution of STORM and also a relatively high copy number of synaptophysin proteins compared to other SV proteins, the structure could not be clearly resolved. This is again due to the high packing density of structures. However, when placing the label within the SV lumen, individual SVs can be resolved as was shown in Figure 3-41. In conclusion, the results of the simulation confirms the real measurements performed on SV clusters as shown in Figure 3-34. Neither discrete actin filaments, nor densely packed vesicles could be clearly resolved by means of the STORM super-resolution technique. Consequently, also the role of actin for SV clustering cannot be discerned with STORM. To avoid such over-estimations of SMLM techniques, the SuReSim software was developed and its use to avoid over-interpretation will be described in the next section.

4.5 Limits of SMLM techniques
The simulations of actin as shown in Figure 3-40 clearly show that for the identification of densely packed structures both the labeling efficiency and the localization precision achievable with SMLM might be limiting. Even in the best case scenario with the direct simulation and therefore lacking imaging artifacts, assuming the best microscope available, a tiny label and 100% labeling efficiency it is impossible to resolve the fine actin network in the membrane of erythrocytes as well as individual vesicles in densely packed regions of the SV cluster.
The extreme variation of binding angles of the label has great influence on the resulting image as can be seen in Figure 3-41. Here, inward bound and membrane-proximal labels resulted in images
that reflected the ground truth models in more detail. However, while outward bound labels and membrane bound labels are easily achieved under experimental conditions, the inward bound label that provided best simulation results might be difficult to achieve. The introduction of inward bound labels via antibodies, would require the mechanical penetration of SVs. Furthermore, with a total length of ca. 16 nm (corresponds to two antibodies), the inward bound antibodies would fill a very high portion of the SV. The advantage of inwardly located labels may arise from the spheric nature of the SVs, yet detection of every small tubular or spherical structure will benefit from a label placed in the lumen of the structure. This example shows that SuReSim can be efficiently used to identify and test new experimental paradigms.

The actin strands (Figure 3-42) and the vesicle cluster (Figure 3-43) both show cases where the interpretation of the experimental data is difficult. For experimental results only the reconstruction would be available. In case of the vesicles a higher labeling efficiency might help to distinguish unspecifically bound background localizations from specific bound signal. The problem to distinguish background from specific signal becomes even more severe when for example small protein clusters are investigated.

The resolution of SMLM techniques is also limited by the dyes that can be used, especially by the photon output (see Figure 3-32). SuReSim can also be used to simulate dyes of different intensity and switching kinetic which might be useful to find the proper dye for an experiment. Equally limiting as the label and imaging properties is the structure of interest itself. In general, dense structures are harder to resolve than expected based on the distances between individual components. For example, the chance of overlapping PSFs, which lead to incorrectly detected PSFs, increases with the density of labels, also the size of the label has to be considered. All limitations of SMLM that are based on the limited labeling efficiency or the label epitope distance pose the same problems for other SR techniques like STED as well.

SMLM is also limited to thin sections. One reason is the high amount of background fluorescence when thick sections are imaged, another that the shape of the PSFs get worse the deeper from within the tissue they originate. Compared to STED this is a strong limitation since images can be recorded up to 120 μm depth using STED. The focal depth for SMLM measurements is usually about 1 μm. Also image acquisition of large areas is problematic due to the long time a single recording takes.

In summary, the results of SMLM experiments has to be carefully interpreted and one should simulate the expected conditions of an experiment beforehand to evaluate whether or not SMLM is the right tool to answer the research question of interest.
5 Conclusion

I assembled and partly designed a 3D-dual color STORM microscope, developed a controlling software for the microscope and wrote a post-processing software package. Based on these components an efficient and robust STORM workflow consisting of image acquisition, reconstruction and post-processing has been established. Using this workflow, measurements of neuronal tissue were performed and showed that SMLM offers a new tool box to investigate protein distributions with a resolution of several nanometers. Nevertheless, super-resolution techniques bring their own constraints.

Noteworthy, the experimental results uncovered that the label type that is used for STORM measurements has a big influence on the image quality. Especially for densely packed structures the label size plays an important role. Also the labeling efficiency and the total number of binding sites available have to be considered. For large structures with a high number of epitopes, like microtubules, this is no big concern but for fine structures, like actin filaments, high labeling efficiencies are needed. Furthermore, experimental data might be over-interpreted, leading to false conclusions about the investigated structure. In summary, the limits of SMLM strongly depend on the structure of interest and the label that is used. It is not possible to make general statements about the limits that hold true for all structures and all labels, but the impact of imaging parameters on the potential outcome of the imaging experiment has to be considered for each application individually.

This is what the simulation software for super resolution data, SuReSim, can deliver. It allows disclosing the limitations that could appear in super-resolution experiments, making SuReSim a valuable tool for the evaluation of the feasibility of specific research questions. In particular, it helps to identify the most critical parameters, before performing time-consuming experiments and can ascertain an economic use of resources. It can also be used to test post-processing workflows even before experimental data is acquired. Results that are difficult to interpret can be validated by performing simulations and comparing the simulated results with results from real experiments. SuReSim is the most realistic simulation tool currently available because it enables users to import arbitrary 3D ground truth structures, simulate realistic labels, behavior of the fluorophores and camera properties. It also provides an instant 3D visualization of the simulated data that can be interactively explored.

Since the model creation can be the most cumbersome part of the simulation process, an increasing set of 3D models from the field of EM microscopy will help to further improve and promote the use of SuReSim.
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7 Declaration

Hereby I declare that in this thesis I presented my original research results. I have written this thesis by myself and marked the sources of any materials previously published or written by another person.

The work has been done under the guidance of Prof. Dr. Thomas Kuner at the Department of Functional Neuroanatomy, University of Heidelberg, Germany and co-supervised by Mike Heilemann at Institute of Physical and Theoretical Chemistry, Goethe University Frankfurt, Germany.

This thesis is being submitted for the degree of Doctor of Natural Sciences at the University of Heidelberg.

Heidelberg, March 2017

Frank Herrmannsdörfer

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