Single-molecule localisation microscopy (SMLM)

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Chapter I.3.c: Single-molecule localisation microscopy

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Abstract

Conventional light microscopy methods have long been thought to be ultimately limited by diffraction of light. According to Abbe's law the achievable resolution cannot reach beyond half the imaging wavelength of around 200nm. The advent of super-resolution microscopy techniques in the last decade has led to a breakthrough in light microscopy, achieving resolution far beyond the diffraction limit.

Single molecule localization microscopy (SMLM) methods exploit the stochastic switching of fluorescent probes, allowing to separate their signals in time and determining their three-dimensional positions with nanometer precision. The advances in SMLM allow to observe cellular structures in unprecedented detail. The method offers the possibility to perform biomolecular quantification and investigate protein oligomerization and clustering on the nanoscale.

This book chapter introduces the physical principles behind SMLM and its implementation, giving an overview of available labeling and switching techniques. The most important parameters to consider for achieving high image quality are explained and common challenges are pointed out. Furthermore, required steps for processing recorded data, as well as visualization and advanced analysis methods will be described.

1. Introduction

The phenomenon of diffraction has long been thought to set an inevitable limit to resolution of light microscopy. According to Abbe's diffraction limit, structures smaller than half the wavelength of light cannot be resolved. The last decades however, have seen developments in fluorescence microscopy that enable to study cellular structures below the diffraction limit.

One of these approaches has been termed single molecule localization microscopy (SMLM). In 2006, different realizations of the technique were developed simultaneously: photoactivated localization microscopy (PALM), fluorescence photoactivation localization microscopy (fPALM) and stochastic optical reconstruction microscopy (STORM). More recent approaches include direct stochastic optical reconstruction microscopy (dSTORM) and point accumulation for imaging in nanoscale topography (PAINT), including DNA-PAINT. These SMLM variants will be covered in this chapter (see reference [1-4] for further information).

The basic idea of all SMLM techniques is to separate the signal of individual emitters in time, which allows to determine their positions with nanometer precision. This is typically achieved by exploiting stochastic blinking or binding phenomena, leaving only a sparse subset of molecules visible at a certain time point. Hence, for image acquisition thousands of individual frames need to be recorded. The raw data is subsequently analyzed, yielding a list of localization coordinates as a final result.

Although SMLM experiments can be performed on modern fluorescence microscopy systems, experimental planning, data collection, visualization and data analysis differ substantially from standard diffraction-limited microscopy. In this chapter, we highlight the particular aspects of SMLM and discuss state-of-the-art solutions to present problems.

2. Principles and Set-ups

2.1 Physical Principles

Principle

All single molecule localization microscopy techniques rely on the stochastic separation of signals from different emitters in time. Fig. 1 depicts the basic idea of SMLM. In each frame, only a sparse

subset of all fluorescent labels are active, so that their individual signals are well separated. Determining the center of each fluorescent signal allows to determine the position of emitters with nanometer precision. Thus, localization coordinates can be assigned to the labels. Typically, thousands of frames are recorded until most labels have been detected at least once. The recorded signals are subsequently fitted and finally, the obtained localizations from all frames are combined to yield a reconstruction of the image. The resolution of the image is not limited by the diffraction of light anymore, but instead by the precision and accuracy of the position estimation for individual emitters.

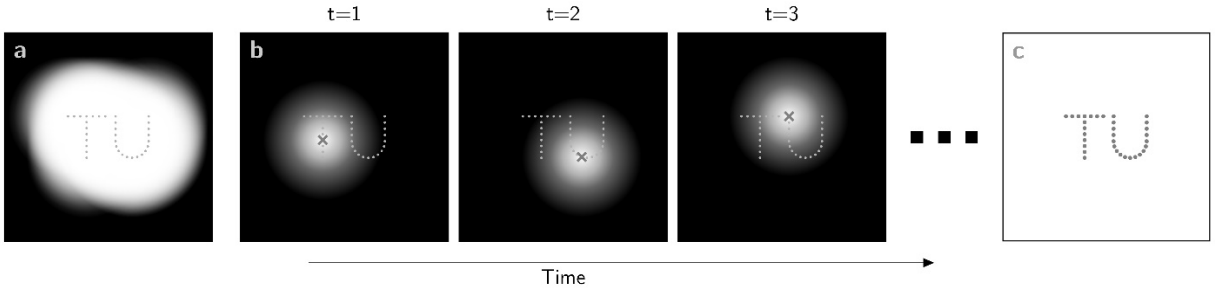

Fig. 1 Principle of single molecule localization microscopy (SMLM).

The structure of interest is labeled with fluorophores. In the diffraction-limited image (a), the PSFs of individual emitters overlap and the structure cannot be determined. For SMLM, the fluorophores switch stochastically between a bright (fluorescent) on-state and a dark (non-fluorescent) off-state. In each frame, only a small subset of all labels is in the bright state, so that their fluorescence signals are well separated and the position of the molecules can be determined with high precision (b). Finally, the obtained localizations from all acquired frames are combined to yield a reconstructed super-resolution image (c).

Methods for separating fluorescent signals in time

The stochastic separation of fluorescent signals of individual emitters in time can be achieved in several ways. In the following, an overview of different methods is given, which are also depicted in Fig. 2.

Switching methods are based on transitions of fluorescent labels between a bright *on*- and a dark *off*-state. The *on*-state is fluorescent and the label can be detected as a signal on the camera chip, whereas the *off*-state is non-fluorescent and does not emit any signal. Stochastic transitions between the bright and dark state are achieved by irradiation with light of appropriate wavelength. The main mechanisms are the following:

- *(Photo-)Activation*: An initially non-fluorescent molecule acquires fluorescent properties. This process can either be induced by irradiation with light of appropriate wavelength (photoactivation) or occur spontaneously by oxidation of the fluorophore.
- *Photodeactivation*: Irradiation with light of appropriate wavelength causes the loss of fluorescent properties of the molecule.
- *Photoconversion*: Irradiation with light of appropriate wavelength leads to a photoinduced shift of the excitation and/or emission spectrum.

Commonly, a combination of these mechanisms is used for imaging. A variety of underlying physical or chemical processes are available, resulting in varying nature of the dark state and degree of reversibility to the bright state. Reversible transitions between the bright and dark state are usually referred to as *photoswitching*, whereas the permanent transition into the dark state is termed *photobleaching*. A broad range of physicochemical mechanisms are responsible for inducing the switching behavior. Photoactivation and photoconversion typically involve a bond cleavage. Possible underlying processes for photoswitching include cis-trans isomerization steps, proton transfer or chemical reactions with extrinsic additives, e.g. redox reactions. The methods PALM and fPALM apply photoactivatable, photoswitchable or photoconvertible fluorescent proteins to achieve switching, while STORM and dSTORM rely on chemically induced blinking of organic dyes.

A different approach for separating the emission of fluorescent labels in time is based on **transient binding events**. It is implemented in PAINT and various extensions of it. Here, fluorophores diffuse freely in the imaging solution. The diffusion times exceed the illumination time for one frame, which leads to spreading of the signal over the camera chip. Hence, unbound fluorophores can only be observed as background fluorescence. Additionally, quenching of fluorophores in the unbound state may suppress their emission. Upon spontaneous binding to the target, the fluorophore is immobilized for at least a few milliseconds and can be detected as a bright fluorescent spot. Due to the stochastic nature of the binding process, the fluorophores in solution bind to the target structures at different times and hence, their signal is separated in time. The binding rate can be adjusted by changing the concentration of the fluorophore in

solution, while the duration of a binding event can be adjusted via the binding affinity of the used label to its target.

The original PAINT approach has been generalized under the acronym uPAINT (universal points accumulation for imaging in nanoscale topography). A main advancement of the principle is DNA-PAINT. Here, short fluorescently labeled oligonucleotides (*imager strands*) diffuse in solution and transiently bind to complementary DNA *docking strands* that are attached to the target of interest. Altering the specific oligonucleotide sequence and its length offers the possibility to conveniently adjust binding strengths. In combination with the adjustment of imager strand concentration, DNA-PAINT thus allows for programmable binding and unbinding kinetics.

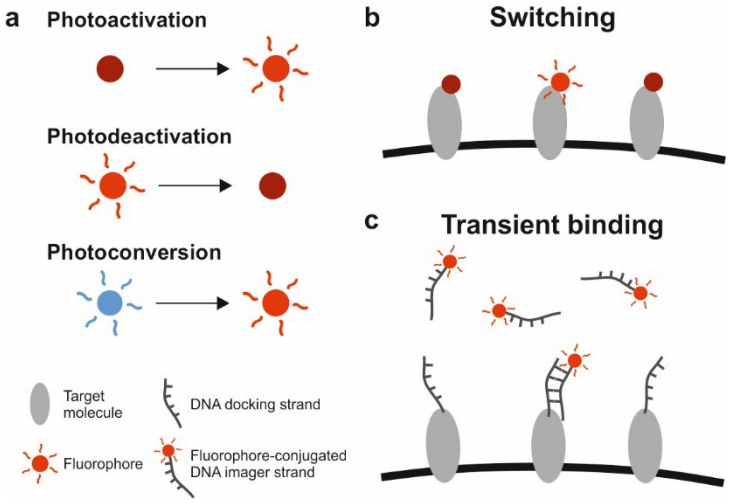

Fig. 2 Methods for separating fluorescent signals in time.

(a) Depiction of (photo-)activation, photodeactivation and photoconversion. In photoactivation and deactivation the fluorophore is transferred from a dark into its bright state, or vice versa, upon irradiation with light. Activation can also occur spontaneously by oxidation of the fluorophore. In photoconversion, the fluorophore is transferred from one bright state into another bright state upon irradiation with light. (b) Switching methods include (d)STORM and (f)PALM. Here, fluorophores are bound to the target molecules. Stochastic switching of the fluorescent labels between a bright and a dark state allows to separate signals in time. (c) Transient binding methods, including PAINT and variants of it, rely on the transient binding of the fluorescent labels to the target of interest. Here, DNA-PAINT is shown. The molecule of interest is labeled with a short DNA docking strand. The complementary imager strands carry the fluorophore and diffuse freely in solution, smearing out their fluorescent over the whole image. Upon binding of an imager strand to a docking strand, the fluorophore is immobilized for a certain time and can be detected as fluorescence signal on the detector.

2.2 Typical Set-ups

Typically, imaging is performed on conventional inverted wide-field fluorescence microscopes modified for single-molecule microscopy. Since more recently, also commercial systems that incorporate options for SMLM imaging are available.

Oil-immersion objectives with high numerical aperture (typically NA>1.4) achieve a high photon collection efficiency and thus, improve localization precision (compare section 4.2). For intra-cellular imaging, also water-immersion objectives are used.

Illumination light needs to be ideally uniform in intensity and of well-defined wavelength. Furthermore, high illumination power is required for fluorophore excitation. Therefore, lasers are taken as light source, which need to match the respective activation and excitation spectra of the used fluorophores. Excitation filters and beam shapers are inserted into the excitation path for optimization of the laser beam. Commonly used laser lines are e.g. 488, 532, 640nm for fluorophore excitation and 405nm for activation. Excitation intensities are typically 0.5-5kW/cm² for excitation and less than 0.1kW/cm² for photoconversion or photoactivation. Appropriate emission filters separate excitation from emission light and thereby improve the signal-to-noise ratio.

Several modes of sample illumination are available for SMLM. Total internal reflection fluorescence (TIRF) and highly inclined and laminated optical sheet (HILO) microscopy can be implemented without the need for further optics. In TIRF microscopy (see also Chapter 3.a), illumination at the critical angle causes an evanescent wave that excites only those fluorophores that are located within approx. hundred nanometers from the coverslip. For HILO, illumination at an angle slightly lower than the critical angle leads to illumination of a thin section of the sample only. Illuminating only a small layer of the sample has the advantage of reducing background form out-of-focus fluorescence, thus increasing the signal-to-noise ratio, as well as reducing out-of-focus photobleaching. Other illumination methods require additional optics, e.g. lattice light-sheet microscopy (see chapter 2b).

The photons emitted from the fluorophores are detected and imaged on a sensitive camera chip. Apart from traditional charge-coupled device (CCD) cameras, further options are available. Electron-multiplying charge-coupled device (EMCCD) cameras amplify the signal in order to increase it above read-out noise and thus allows for imaging with very high frame rates. The more

recent development of scientific complementary metal-oxide-semiconductor (sCMOS) cameras is a cheap and even faster alternative to EMCCDs. The pixel size of the camera is typically chosen to be around 100nm, which optimizes localization precision (compare section 4.2).
Acquisition times for a single frame range from 1-20ms for STORM or PALM, and up to 100-500ms for PAINT approaches, due to the longer duration of transient binding events needed compared to fluorophore blinking. Typically, thousands of frames are recorded for one region of interest. Therefore, high computer performance and large memory capacity are needed to cope with recorded data sets of up to several gigabytes.

3D setups

For conventional 2D microscopy, the PSF is axially symmetric. The signal of a fluorophore slightly above and below the image plane cannot be distinguished and thus, the extraction of 3D information is limited. In order to enable localization in the third dimension, the axial symmetry has to be broken. This can be achieved by various methods that rely either on recording the fluorescence of emitters in multiple channels simultaneously (e.g. interferometric approaches) and/or modifying the shape of the PSF by introducing additional optical elements in the light path (e.g. a cylindrical lens for astigmatism). An overview of 3D techniques can be found in [5].

3. Biomedical Relevance

3.1 Application Range

Single molecule localization microscopy techniques yield super-resolved images of cellular structures at the nanometer scale. At the present, SMLM is commonly applied to single cells or an aggregation of a few cells only. The method allows for studying the spatial arrangement of molecules in the cell plasma membrane or within the cell and investigating potential interactions between different types of molecules. As the method is based on light microscopy, cellular structures may be imaged and investigated in their natural environment, which is one of the primary advantage of SMLM in comparison to other high-resolution techniques. Structures being imaged and characterized by SMLM include the nuclear pore complex and actin structures (Fig. 3). Studies based on SMLM have also led to the discovery and extensive characterization of

protein nanoclusters, whose existence has, however, been disputed due to the difficulty of appropriately accounting for imaging artifacts (see section 5.4).

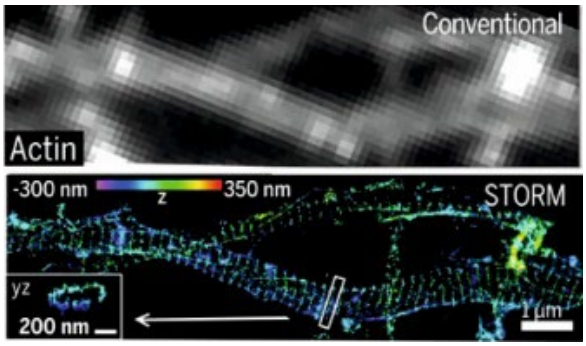

Fig. 3 Representative image obtained by SMLM.

Diffraction-limited (top) and 3D-STORM (bottom) images of actin in axons. The STORM image reveals the arrangement of actin in a periodic ring structure. Figure with permission from [1].

As the structure of interest needs to be labeled with a fluorophore, typically only one or a few specific molecules are imaged at once (see also section 4.8). Recently however, imaging of even up to a hundred targets on the same sample has been reported.

The high spatial resolution achievable with SMLM comes at the cost of temporal resolution. For obtaining a single image, thousands of individual frames need to be recorded, resulting in long acquisition times of several minutes to hours. As individual molecules are detected at separate time points, any movement of the structure during imaging will lead to distortions. Hence, SMLM is commonly performed with fixed cells. Live-cell imaging SMLM has been reported previously, however, it imposes major challenges, such as the availability of suitable fluorescent probes, limits on temporal resolution and reduced spatial resolution due to sample movement during image acquisition.

3.2 Sample Preparation

In order to perform an SMLM experiment, the sample has to be prepared appropriately. In the following, the most important steps in the preparation procedure will be discussed.

Fixation

As mentioned above, SMLM is typically performed in fixed cells. Fixation is usually achieved by means of chemical fixatives, typically paraformaldehyde and/or glutaraldehyde, which cross-link

cellular proteins by covalent chemical bonds. Crucial for any fixation method is the preservation of structural organization at the nanoscale. Applied fixation protocols should be optimized in order to reduce any induced structural artifacts.

Labelling

For SMLM applications, prerequisite for any fluorescent labeling technique is to enable switching between a bright and a dark state. The labeling of choice depends on the target structure and label properties required for specific applications, for example low cell toxicity for live-cell imaging or cell permeability for labeling of intracellular targets. Importantly, the original properties of the molecules of interest should not be affected. For quantification purposes, a well-defined stoichiometry of labeling is crucial. Overall requirements demanded from any fluorescent probe include high labeling efficiency, small size of the label and specificity of the binding, in order to ensure that the labeling correctly represents the structural organization of the molecules of interest. Furthermore, labels need to exhibit favorable fluorescent properties: particularly, on-off switching contrast and number of switching cycles influence the quality of the obtained image. Most importantly, a high photon budget, i.e. brightness, is a prerequisite for achieving optimal localization precision in SMLM (compare section 4.2). In the following, we will give a short overview of different types of labeling methods.

Photoactivatable **fluorescent proteins (FPs)** fused to the protein of interest ensure specific targeting. Commonly, they are derivatives of the green fluorescent protein (GFP). Photoactivatable, photoswitchable or photoconvertible FPs allow for SMLM applications. However, with a size around 2-5nm FPs are rather larger and it has to be ensured that original protein functionality is not perturbed. Moreover, detection efficiency can be reduced due to the presence of not fully matured FPs as well as photobleaching before the actual detection step. Importantly, the brightness of FPs is generally worse compared to organic fluorophores, which affects localization precision. Furthermore, an obvious disadvantage of FPs is the exclusive applicability to proteins.

Organic dyes (e.g. rhodamine and cyanine dyes) represent another group of fluorescent labels. Two commonly used families of dyes are the Alexa Fluor and ATTO dyes, available at various excitation and emission spectra. These synthetic fluorophores have a rather small size of around 1nm, high quantum yield and extinction coefficient. For application in SMLM, a stable off-state

together with appropriate photoswitching rates of the dye are required. The probes typically cycle between their bright and dark state many times before photobleaching. A disadvantage of organic dyes compared to FPs is the need for a chemical labeling procedure. Organic dyes may be attached to the molecule of interest via immunofluorescence (antibodies or nanobodies), enzymatic protein tags (SNAP-, CLIP-, Halo-tags) or click chemistry, which offers labeling of various cellular structures, including proteins, nucleic acids, glycans and lipids. Moreover, certain cellular structures can be targeted with small and very specific labels, e.g. phalloidin in the case of actin, and paclitaxel for labeling of microtubulin.

For DNA-PAINT, the structure of interest is not directly labeled with a fluorescent probe, but bound to a short oligonucleotide strand, the docking strand, for example via an antibody or an enzymatic protein tag. The fluorophores are coupled to complementary oligonucleotide strands, the imager strands, as part of the imaging solution.

Imaging buffer

In a typical SMLM experiment, cells are seeded on a microscopy glass coverslip and covered in imaging buffer. Imaging of organic dyes requires special buffers for inducing blinking and control of the on-off switching rates. These buffers usually include thiol and an oxygen scavenging system in order to improve switching behavior. Buffer conditions should be optimized for achieving best imaging conditions. For fluorescent proteins in contrast, cells can be embedded in standard water-based saline solutions without the need for any additional ingredients. In the case of DNA-PAINT, the imaging solution is also based on a saline solution. However, it additionally needs to contain the fluorophore-conjugated imager strands, which diffuse freely in the solution.

4. Parameters of Image Quality

As SMLM requires careful preparation of the sample as well as complex experimental procedures and data analysis, a wide range of parameters influence the final quality of the obtained images. In this section, we will give an overview of the most important aspects to take into account. Fig. 4 depicts the influence of several parameters on the final image quality.

4.1 Spatial Resolution

For localization microscopy, the term *resolution* used in the conventional theory of Abbe and Rayleigh has to be redefined [6]. In the context of SMLM, spatial resolution is commonly reported as the precision achieved in the localization of a single emitter (see section 4.2). However, resolution is not only limited by localization precision, but also by labeling density and detection efficiency. In fluorescence microscopy in general, the sample is labeled at discrete sites, i.e. the molecules of interest. If labeling is too sparse, structural details cannot be resolved, even in case of optimal localization precision. Here, often the Nyquist-Shannon sampling theorem is taken as a guideline, which states that neighboring localized emitters need to be closer than half of the smallest structural feature that can be resolved. However, for structures that do not show a continuous boundary, e.g. individual molecules in the cell membrane expressed at low levels, the final image will inherently be sparse. Thus, the reliability of obtained information also depends on the specific features of the structure under investigation.

4.2 Localization Precision

The fundamental concept behind SMLM is to determine the position coordinates of isolated emitters from their PSF image. However, the position can only be estimated with limited certainty. The standard deviation of this error distribution is referred to as the *localization precision*. It is influenced by several parameters, including the width of the PSF, shot noise, and background noise. Various theoretical formulas have been derived to estimate localization precision based on these parameters [5]. An estimate of achieved precision may be acquired experimentally by repeatedly imaging and localizing the same emitter, and determining the spread of position coordinates.

The main impact on localization precision arises from shot noise, which originates from the particle nature of light and scales with the square root of the number of detected photons N. The photon count depends on illumination time, wavelength of illumination and photophysical properties of the fluorophore, including extinction coefficient, quantum yield and photostability. Therefore, fluorescent probes with high photon output are desired for SMLM applications.

Background signal primarily arises from residual cellular autofluorescence, and from Rayleigh or Raman-scattered light. Careful choice of emission filters, and selective excitation of the focal plane (see section 4.4.) helps to reduce these contributions. Moreover, dark current including thermic noise and cosmic noise adds to background. This type of noise causes a Poisson distributed background and increases with exposure time. Cameras are typically cooled in order to minimize dark current contributions to the image. A further source of noise is camera read-out noise, which arises from the electronics when reading out the image of a camera chip and is independent of the detected photon number. Read-out noise has been dramatically reduced by the introduction of EMCCDs and sCMOS.

In addition, localization precision is influenced by the pixel size. On the one hand, smaller pixel size of the camera chip allows to better resolve the PSF. On the other hand, for smaller pixel size, fewer photons are detected per pixel and the signal ultimately gets lost in detector noise. Hence, the optimal pixel size typically corresponds to 100 to 160nm in the object plane.

4.3 Localization Accuracy

The aim in SMLM is to correctly determine the position coordinates of molecules of interest. However, correct position estimation is not only affected by limited precision, but may also be impaired by nonzero *localization accuracy*, which describes the deviation of the mean measured position from the true position coordinate of the target molecule.

In general, it is not the molecule of interest itself that is detected in SMLM techniques, but a fluorescent probe attached to it (see section 3.2). A bias in localization may arise from the finite size of fluorescent probes themselves, in particular for larger probes like fluorescent proteins. Moreover, linker moieties that connect the target to the fluorophore, e.g. primary and secondary antibodies, can lead to displacements of the fluorophore of up to 10-20nm in a random direction.

Furthermore, fluorophores are dipole emitters and hence exhibit asymmetric emission patterns. If a fluorophore's dipole orientation is fixed and cannot rotate freely, this will lead to distortions of the PSF and thus may cause considerable localization inaccuracies when using common estimators.

4.4 Sample Background

Background fluorescence may arise from cellular autofluorescence, dirt particles, unspecifically bound labels and residual unbound fluorophores. All these factors add noise to the image and hence, decrease the localization precision. Furthermore, signal that is not emitted by fluorophores bound to a target molecule may lead to false positive localizations.

Specialized illumination approaches exist that suppress out-of-focus signal and thus, reduce background fluorescence. Both TIRF and HILO microscopy allow for a confined excitation region and yield an increased signal-to-noise ratio and image contrast. A more sophisticated approach to achieve selective illumination is provided by light-sheet fluorescence microscopy (chapter 2.a and 2.b).

In order to minimize false positive localization, the obtained localizations can be filtered in a post-processing step (see section 5.2). This allows to discard localizations that show characteristics that are untypical for the fluorescent probe used, e.g. very high brightness values, large spread of the signal or enduring on-times.

4.5 Sample Motion

SMLM requires the acquisition of thousands of individual frames for obtaining a final reconstructed image. For achieving reliable images, it needs to be ensured that the sample stays as static as possible during the imaging procedure. However, sample movement may be present, which leads to distortions of the image, especially for prolonged imaging times.

Residual diffusion of molecules within the sample may occur due to insufficient fixation. Additionally, sample movement relative to the detector due to mechanical instabilities of the microscope stage and thermal drift may distort the image, especially for prolonged imaging times. This stage drift can be compensated for by means of fiducial markers or based on cross-

correlation (see section 5.2). Furthermore, waiting times before beginning the measurement reduce the effect of initial mechanical relaxation.

4.6 On-off Switching and Blinking

The basic principle of SMLM techniques is to separate the signal from individual fluorescence emitters in time. In each frame, only a sparse subset of fluorophores is in its fluorescent on-state, while all others are in the off-state. Imaging conditions need to be adjusted in order to achieve an optimal density of active emitters. If this density is too high, the signals of individual emitters will overlap and impede accurate localization. On the other hand, too few active emitters will reduce the obtained number of localizations per frame and hence, either decrease detection efficiency or require an extremely high number of recorded frames. On- and off-switching rates depend on the illumination protocol, in particular illumination time and power, but are also influenced by buffer conditions.

Ideally, every labeled target molecule is in its on-state in exactly one frame during the whole acquisition procedure and hence, detected exactly once. However, if on-times exceed the acquisition time for a single frame, fluorophores will be detected in multiple consecutive frames. Moreover, fluorescent probes typically switch repeatedly between the on- and the off-state, resulting in overcounting. Organic dyes typically show extensive and complex blinking behavior. The problem of blinking-induced overcounting is further increased by the finding that photophysical properties of fluorescent dyes highly depend on their local nanoenvironment, which hampers corrections based on blinking statistics. Blinking is less pronounced for photoactivatable fluorescent proteins, nevertheless, also FPs may transition into prolonged dark states and subsequently return into the bright state. This aspect has to be carefully considered, especially in quantitative analysis and interpretation of the data (see section 5.4).

4.7 Temporal Resolution

In SMLM, high spatial resolution is traded for temporal resolution. Typically, imaging is performed in fixed cells, i.e. at a single time point only. For live-cell imaging, the achievable temporal resolution mainly depends on the blinking behavior of the fluorescent probe and its photophysical properties, in particular quantum yield. Moreover, frame rates are limited by camera read-out

speed. The overall time required for obtaining an individual image results from the acquisition time for a single frame times the total number of frames that are recorded. The development of faster cameras and algorithms that enable localization in images with substantial amount of overlapping emitters may further increase the speed of imaging acquisition and thus, enable advanced live-cell imaging.

4.8 Multi-color Imaging

In many biological questions, it is not sufficient to investigate a single type of biomolecule in the cell only, but the interaction of several different ones. This aim can be achieved by multi-color imaging.

Conventionally, multi-color imaging in SMLM is implemented by the use of spectrally distinct dyes, each of which targeting a specific molecule of interest. The different types of fluorophores can either be excited and imaged alternatingly in the same detection path, or their respective emission can be split into different color channels and imaged on separate regions of the camera chip. Color channels can be aligned by means of multi-color beads, which allows also to correct for chromatic aberrations. Spectral overlap limits the number of targets that can be imaged simultaneously to about three. In case of (d)STORM, buffer conditions have to be optimized dependent on the specific fluorophore, which further complicates imaging of multiple targets.

A major advancement towards multi-color imaging has been enabled by Exchange-PAINT. Based on DNA-PAINT, this method allows for imaging a large number of different targets, only limited by the number of orthogonal DNA docking strand sequences. The idea is to repeatedly introduce and wash out imager strands specific for a single target until all desired targets have been imaged. The separated imaging steps allow to assign a pseudo-color to each target and obtain a multiplexed final image. For Exchange-PAINT, a single dye and a single laser source are sufficient. Hence, a dye with optimal photophysical properties can be selected for imaging of all targets. Moreover, the method circumvents the problem of chromatic aberrations.

4.9 Reference Structures

Directly assessing the quality of localization microscopy data has often proven to be difficult, especially for imaging of *a priori* unknown structures. The achievable localization precision can

either be estimated via theoretical calculations or experimentally (see section 4.2). For parameters like labeling and detection efficiency however, a direct assessment is very challenging or even impossible.

Therefore, robust test samples are commonly used as a reference in order to judge the quality of SMLM data and optimize imaging conditions. Natural biological samples of a well-defined structure include the nuclear pore complex, centrosomes, clathrin-coated pits and cytoskeleton structures like actin filaments and microtubules. Moreover, DNA origami provide artificial samples that can be freely designed to exhibit a specific structure of choice. As a major drawback, however, obtained results often cannot be transferred directly to the target of interest, as parameters like labeling efficiencies and geometry may possibly differ from the reference sample.

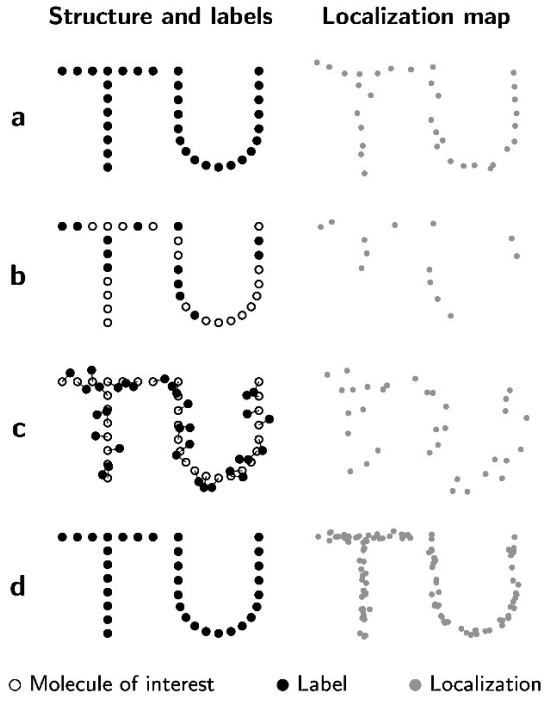

Fig. 4 Influence of imaging parameters on the reconstructed image.

The left column shows the actual localization of the protein molecules (circles), with full and open circles indicating proteins carrying a detectable or no detectable label, respectively. The obtained localization map is shown in the right column. Due to localization errors, the localizations are slightly displaced from the true molecule positions (a). The structure is distorted or misrepresented by decreased labeling efficiency (b), label displacement (c) or overcounting (d).

5. Data Processing and Visualization

An essential part of SMLM is the post-processing of the recorded raw image data in order to obtain the reconstructed final image. In the following, we will give an overview of available localization methods and software, criteria for filtering obtained localizations, and ways to visualize obtained data. Finally, we will describe advanced analysis methods for biomolecular quantification and characterization of possible clustering.

5.1 Localization

Raw SMLM data typically consists of thousands of individual frames. For each frame, the blinking events have to be analyzed and the positions of active emitters need to be determined with high precision and accuracy. Difficulties that have to be faced in the analysis include background noise, uncertainties in the size of the point spread function and possible aberrations. Localization algorithms need to process large amounts of data, typically in the range of gigabytes for a single image. Often, demands for speed and precision of the localization compete, and the applied algorithm or software package has to be selected carefully in order to fulfill the specific needs of the application. An evaluation and comparison of a broad range of available localization software packages both for 2D and 3D SMLM was performed by Sage et al. [7].

As a first step in the analysis, active fluorophores have to be identified. For this, threshold and rejection parameters are set in order to distinguish fluorophores from background and avoid fitting of any signals or peaks that likely do not arise from the fluorescent probes. Subsequently, the localization coordinates of the detected fluorophores have to be determined. In the simplest case, the centroid of the signal can be taken as an estimate for the emitter position. Another commonly applied method is Gaussian fitting of the signal with a least-squares or maximum likelihood approach. Also more complex PSF models can be employed, including theoretical ones based on the laws of diffraction, and experimentally acquired PSF models. Algorithms for 3D localization determine the axial position from characteristics like the shape of the PSF (compare section 2.2).

In general, the density of simultaneously active emitters should be low enough to ensure that individual signals are well separated. However, as blinking is stochastic, it may occur that neighboring fluorophores are active at the same time, leading to insufficient spatial separation of

their signals. Moreover, a high density of active emitters might be desired as it allows for faster acquisition and thus, higher temporal resolution. Specific algorithms exist that allow for multi-emitter fitting, i.e. the fitting of partially overlapping signals.

5.2 Filtering and Corrections

After the blinking events have been analyzed, the resulting list of localizations can be further processed. Commonly, the list is filtered in order to remove localizations that do not fulfill certain quality criteria. Localizations can be restricted to those signals that exhibit a minimum number of photons and hence, a certain localization precision. Moreover, signals with bad least square fitting values can be discarded. Filtering on the width of the PSF provides a form of optical sectioning, as this leads to selection of only those emitters that are in close proximity to the focal plane. Events that occur over a long period of consecutive frames may be removed, as they often do not arise from fluorescent probes. Density filtering discards localizations that are isolated and hence, presumably outside the structure of interest. However, depending on the structural arrangement of the target, this approach may also remove correct localization data. As another post-processing step, localizations from the same emitter detected in different frames can be merged based on spatial and temporal proximity. This reduces the problem of multiple detection of the same emitter (compare sections 4.6 and 5.4).

The obtained localization coordinates further need to be corrected for drift (see section 4.5). This can either be achieved based on fiducial markers or cross-correlation. In the first case, fiducial markers such as fluorescent beads or gold nanoparticles are inserted into the sample and recorded together with it. The markers are tracked and their trajectories are used to retrieve the drift-corrected localizations. Drift correction based on cross-correlation is feasible if the target is highly structured. Partial images reconstructed from sub-sequences of the recorded data can then be cross-correlated in order to determine the drift.

For multi-channel imaging, additional corrections are required in order to account for the shift, stretch and possible distortion between the channels. Particularly, for multi-color imaging, chromatic aberration needs to be corrected for. Registration of the individual channels can be performed based on multi-spectral fiducials, which are detectable in all channels.

5.3 Visualization

Conventional microscopy yields a pixelated image, with pixel values according to the recorded intensities. However, SMLM techniques instead yield a list of localization coordinates and hence, different means of visualizing the recorded data are required.

A simple option is to create a scatter plot, in which each localization is represented by one mark. This type of visualization, however, is less intuitive as it yields an image quite different from conventional ones. Therefore, other visualization methods have been suggested. One possibility is to define a pixel grid and count the number of localizations in each pixel in order to generate a histogram. Arising binning artifacts can be reduced by calculating multiple histograms for different bin positions and averaging the results, yielding so-called average shifted histograms. Another approach is Gaussian rendering, which takes account of different localization precisions for individual observations. Here, each localization is represented as a Gaussian, with the mean at the determined position coordinate and a width according to the localization precision. Options for visualizing three-dimensional data include encoding the z-position in a 2D plot by color, plotting individual sections of the data separately, or generating a 3D plot.

Notably, any visualization method is based on the determined localization coordinates, which do not correspond to the exact fluorophore positions, and to an even lesser extent to the true positions of the molecule of interest. This fact must not be neglected for further analysis and interpretation of the obtained images.

5.4 Advanced Analysis

SMLM is fundamentally different from other imaging techniques, as it yields a list of localization coordinates instead of a pixelated image. This feature enables the opportunity for coordinate-based analysis methods that allow to extract even more detailed information than can be obtained from the mere reconstructed image. Localizations provide intrinsic information about the number of target molecules, allowing for quantitative biology. This includes the determination of protein copy numbers or the number of subunits of protein oligomers, which crucially influence biological function. In addition, the interactions between molecules of the same or different types may be analyzed via coordinate-based colocalization analysis. Moreover, clustering of proteins can be identified and characterized.

Ideally, every molecule of interest is detected exactly once during image acquisition, allowing for direct quantitative analysis of localizations. However, several problems need to be taken into consideration that may hamper correct quantification: (i) *Labeling efficiency*. Target molecules may not carry a fluorescent probe or may not be detected during the imaging procedure. Decreased labeling efficiencies lead to undercounting. (ii) *Stoichiometry of labeling*. Ideal labeling for quantification purposes is one to one, i.e. each molecule carries one fluorescent dye only. Multiple dyes per molecule, as is the case for labeling with antibodies, may increase detection efficiency and fluorescent signal, but are less suitable for quantification purposes. (iii) *Blinking of fluorescent probes*. SMLM relies on the stochastic switching of fluorophores between the on- and off-state. Reversible blinking leads to multiple detections of the same molecule and hence, causes overcounting artifacts.

A simple approach to account for overcounting artifacts is to merge localizations based on spatial and temporal proximity. However, results strongly depend on user-defined thresholds and the method is not suitable to account for complex blinking behavior including long dark times.

Therefore, more advanced methods are based on probability distributions of the number of single-molecule detections, which allow counting of molecules independent of knowledge about exact mechanisms of fluorophore photophysics. These blinking statistics may be determined by performing imaging with sparse labeling, leading to well-separated single molecule signals that can subsequently be analyzed. Notably however, photophysical properties of fluorescent dyes, including blinking, highly depend on their local nanoenvironment. Quantitative PAINT (qPAINT) takes advantage of the programmable and predictable kinetics of transient binding events between imager and docking oligonucleotide strands in order to correctly count target molecules.

One major focus in SMLM applications is the analysis of clustering. Studies performed with SMLM have reported nanoclustering of various proteins in the cell membrane. More recently, however, notes of caution were raised due to the finding that overcounting of single protein molecules due to fluorophore blinking may easily be misinterpreted as the presence of protein nanoclustering (compare Fig. 4d). A discussion of approaches that try to identify and characterize clustering can be found in [8].

Another area of advanced analysis is single particle averaging, which is feasible if the target of interest is expected to have a well-defined structure. Hundreds to thousands of images of

identical particles are acquired, and subsequently registered and averaged. Thus, noise is suppressed and the effects of under- and overcounting can be diminished. As an example, particle averaging has successfully been applied to reveal the detailed structure of the nuclear pore complex [9].

6. Conclusions

6.1 Strengths and Limitations

Single molecule localization microscopy circumvents the diffraction limit of light, achieving high spatial resolution of down to 1nm. This allows to study cellular structure and organization in unprecedented detail and to gain deeper insights into cellular mechanisms on the single-molecule level.

However, the high spatial resolution comes at the cost of a loss in temporal resolution. Furthermore, SMLM data have to be analyzed and interpreted with care: Reduced labeling efficiency leads to undercounting, while labeling stoichiometry and fluorophore blinking are causes for overcounting and thus, induce clustering artifacts.

Reference structures provide a way to assess parameters such as localization precision, labeling density and artifacts, verify the reliability of obtained images and optimize imaging procedures. As a major drawback however, the results cannot be directly transferred to other molecules of interest. Alternatively, the reliability of recorded data may be assessed by comparison of the obtained localization map with the diffraction-limited image.

6.2 Future Developments

Current developments promise even further improvements of SMLM techniques. Exploring new labeling strategies may lead to higher labeling efficiency together with high specificity for the target molecule, which reduces unspecific background. Advancing the photophysical properties of fluorescent probes promises an increased photon-yield, thus achieving even better localization precision. The development of sCMOS cameras has already led to a substantial improvement in imaging speed. High imaging rate together with optimal fluorophore brightness paves the way for live-cell imaging applications.

Great potential lies in the combination of SMLM with other imaging techniques. In correlative light and electron microscopy (CLEM), both the high spatial resolution of EM and molecule-specificity of fluorescence microscopy are accomplished.

At the present, SMLM is commonly applied to single cells or aggregations of a few cells only. Improvements in computational memory and power will allow to store, transport and process large amounts of data, which enables the acquisition of images from larger regions of interest. As techniques, hardware and algorithms improve, three-dimensional imaging of multi-cellular complexes or even whole tissues may be envisioned. Furthermore, machine-learning approaches may lead to advanced image processing and analysis tools.

7. Acknowledgements

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8. References

[1] Sigal Y M, Zhou R, Zhuang X. Visualizing and discovering cellular structures with super-resolution microscopy. Science. 2018; 361:880-7.

[2] Birk U J. Super-Resolution Microscopy. 1st ed. Weinheim: Wiley-VCH; 2017.

[3] Kubitscheck U. Fluorescence Microscopy. 2nd ed. Weinheim: Wiley-VCH; 2017.

[4] van de Linde S, Heilemann M, Sauer M. Live-cell super-resolution imaging with synthetic fluorophores. Annu Rev Phys Chem. 2012; 63:519-40.

[5] Deschout H, Cella Zanacchi F, Mlodzianoski M, Diaspro A, Bewersdorf J, Hess S T, Braeckmans K. Precisely and accurately localizing single emitters in fluorescence microscopy. Nat Methods. 2014; 11(3):253-66.

[6] Nieuwenhuizen R P, Lidke K A, Bates M, Puig D L, Grunwald D, Stallinga S, Rieger B. Measuring image resolution in optical nanoscopy. Nat Methods. 2013; 10(6):557-62.

[7] Sage D et al. Super-resolution fight club: assessment of 2D and 3D single-molecule localization microscopy software. Nat Methods. 2019; 16(5):387-95.

[8] Baumgart F, Arnold A M, Rossboth B K, Brameshuber M, Schutz G J. What we talk about when we talk about nanoclusters. Methods Appl Fluoresc. 2018; 7(1):013001.

[9] Szymborska A, de Marco A, Daigle N, Cordes V C, Briggs J A G, Ellenberg J. Nuclear Pore Scaffold Structure Analyzed by Super-Resolution Microscopy and Particle Averaging. Science. 2013; 341.