Doctoral Thesis

Superresolution microscopy method development and application for the study of mammalian septin filaments

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SUPERRESOLUTION MICROSCOPY METHOD
DEVELOPMENT AND APPLICATION FOR THE STUDY
OF MAMMALIAN SEPTIN FILAMENTS

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by
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Abstract

This thesis provides novel methods and imaging strategies for superresolution fluorescence microscopy and their application to the study of septins. While recent techniques in fluorescence imaging have bridged the gap in resolution between light and electron microscopy, in practice, their performance is mainly limited by fluorophore photophysics and the specificity and density with which cellular molecules are labeled. The precise determination of the spatial relationship between different cellular constituents brings further challenges to the sample labeling and additional technical difficulties.

Focusing on these key problems, the work presents a simple and robust two-color labeling and imaging strategy. Therein, recombinantly expressed constructs tagged with GFP and RFP-derivatives serve as epitopes for small, high-affinity binders coupled to bright fluorescent probes. The small binders offer a superior accuracy in delivery of the fluorophore and higher accessibility of their epitopes in the dense cellular environment. These advantages were combined with spectral-demixing acquisition towards chromatic aberration-free and image registration-free dual color superresolution imaging. The presented method overcomes many serious technical difficulties and provides high-quality two-color imaging with a resolution close to the limit achieved in one color for virtually any combination of the widely available GFP and RFP-derived fusion constructs. In a further step, this dual color method was extended to the third dimension to establish a novel two-color 3D imaging technique.

The developed methods were employed to study the cellular architecture of mammalian septin structures. Septins are a family of filament-forming GTPases which are essential during cell division, polarization and migration. In vitro studies suggest two stages of septin assembly: (i) individual molecules assemble into
nonpolar, rod-like, hetero-oligomeric complexes, and (ii) these assemble end-to-end to form filaments which form the basis of higher-order structures. However, how the assembly of higher order structures takes place \textit{in vivo} remains elusive. The main biological questions posed in this work are: (i) how are complexes arranged in functional filaments (ii) what are the dynamics of filament assembly and (iii) what is the minimal unit of exchange within higher-order structures.

State-of-the-art microscopy methods were used to visualize the positions of individual complexes and subunits in filaments of mammalian cells to provide novel insights into their assembly. Furthermore, the exchange unit of septin filaments in their native environment was characterized bringing progress into understanding the principles governing rearrangements and dynamics of the septin cell cortex.
Zusammenfassung

wendet. Septine umfassen eine grosse Familie von Filament-bildenden GTPasen, welche für die Zellteilung, -polarisierung und -migration essentiell.

*In vitro* Studien schlagen zwei Phasen vor für die Assemblierung von Septinen: (i) Einzelne Moleküle fügen sich zu symmetrischen, stabförmigen hetero-oligomeren Komplexen zusammen, und (ii) diese fügen sich wiederum End-zu-End zu längeren Filamenten zusammen welche die Grundbasis für höherrangige Strukturen bilden. Die Regeln dieser Assemblierung *in vivo* sind nur wenig verstanden.

Die zentralen biologischen Fragestellungen dieser Arbeit sind: i) wie sind die Komplexe und funktionalen Filamenten angeordnet ii) was ist die Dynamik der Assemblierung von Filamenten iii) was ist die kleinste Einheit deren Austausches innerhalb höherrangiger Strukturen.

Fluoreszenzn-mikroskopische Methoden auf dem neusten Stand der Technik wurde benutzt um die Anordnung einzelner Komplexe und Untereinheiten in Septin Filamenten von Säugerzellen zu untersuchen. Neue Einsichten in die Assemblierung funktioneller Komplexe wurden herbeigeführt.

Ferner wurde die Einheit des Austausches in der nativen Umgebung von Filamenten charakterisiert, um weiteren Fortschritt im Verständnis zu den Gesetzmässigkeiten erbracht, welche die Umordnung und Dynamik des Septin Zellkortexes bestimmen.
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Part I

Introduction
Chapter 1

Mammalian septins

Septins were discovered in the 1970s in the budding yeast *Saccharomyces cerevisiae* cell cycle mutagenesis screen [38], the name derives from their specific localization at the septum of mother and bud [9]. Septins are GTP-binding proteins that are ubiquitous in organisms ranging from yeast to humans, though they are absent in higher plants [75, 78]. The number of septin genes present varies significantly between different organisms, with two in *Caenorhabditis elegans*, seven in *S. cerevisiae*, five in *Drosophila melanogaster* and 13 in humans [54, 74]. Septins are considered to be a component of the cytoskeleton due to their tendency to assemble in specific combinations into hetero-oligomeric complexes and further higher-order structures such as filaments that form rings, meshes, hourglasses or gauzes. Polymerized septins are usually associated with the plasma membrane, actin filaments or microtubules. In these assemblies, septins function in key cellular processes by providing diffusion barriers, scaffolding signaling proteins, remodeling the cellular cortex and presumably performing other tasks as well. Several human diseases including cancer, neurodegenerative disease, infertility and multiple pathologies in diverse human organs are associated with aberrant septin function, overexpression, or mutations [21, 85].
1.1 Mammalian septin gene family

In mammals, there are 13 septin genes encoding for the protein products SEPT1-SEPT12 and SEPT14 that were classified into four subgroups according to sequence homology: the SEPT2 group (SEPT1, 2, 4 and 5), SEPT3 group (SEPT3, 9 and 12) the SEPT6 group (SEPT6, 8, 10, 11 and 14) and the SEPT7 group (SEPT7) [55] (Figure 1.1 A). The complexity of the septin family arises from the existence of multiple splice isoforms of some members, which results in the expression of over 30 peptides in mammals with sizes ranging from 30 to 65 kDa.

All polypeptides of mammalian septin family have a GTP-binding domain, polybasic region, variable N-terminal region and some septins contain a coiled-coil region in their C-termini (Figure 1.1 B).

As all P-loop GTPase proteins, like kinesin, myosin and ras proteins, septins share a GTP-binding domain with conserved G1 to G5 motifs in the GTPase core [78, 113]. Of these, the G1 and G3 motifs are conserved up to 98% and 80% and are responsible for the binding of all three phosphates of the GTP nucleotide. The G3 motif, in addition, can bind Mg$^{2+}$. The G4 motif is 90% conserved and is important for conferring GTP binding specificity over other nucleotides. The G2 and G5 motifs are not conserved throughout all members of the P-loop GTPase class. GTP binding and hydrolysis cause conformational changes that lead to the stabilization of septin-septin interactions [47]. SEPT6 group members pose an exception, as they lack the ability to hydrolyze GTP [97].

Figure 1.1: Basic properties of septin family proteins. (A) Homology-based septin subgroups, which are named by founding members. Adapted from Ref. [54]. (B) Scheme of conserved septin domains. Adapted from Ref. [27].
The GTP-binding domain is flanked by the polybasic region on the N-terminus and septin unique element (SUE) on the C-terminus. The polybasic region is 90% conserved in all septins and was shown to bind phospholipids [12, 119]. The SUE is a highly conserved motif that distinguishes septins from other GTPases, however its function is yet unknown.

The C-terminal domain is variable in length depending on the septin group, e.g. SEPT3 group members lack a predicted coiled-coil region in their C-termini. The C-terminal domain was shown to mediate septin-septin and septin-protein interactions [78] and many post-translational modification sites were identified in this region [41]. The N-terminal domain of septin proteins varies between group members and different isoforms of individual proteins.

1.2 Mammalian septin complexes

Since their discovery, septins have been considered as components of the cytoskeleton and are present in cells as filamentous assemblies, which are associated with the plasma membrane, F-actin or microtubules. The cytosolic septin pool is thought to consist exclusively of small hetero-oligomeric complexes [8, 91] and no cellular function for septin monomers has been proposed so far.

As shown by electron microscopy, recombinant and immunoaffinity-purified mammalian septins self-assemble into stable rod-shaped structures (Figure 1.2 A) of 7-9 nm width and heterogeneous length distribution where the majority is ~27 nm long and a less frequent group is of ~35 nm length [43, 60, 91, 96]. These rod-like assemblies, prevalent under the high salt conditions, are termed septin complexes. In low ionic strength septin complexes polymerize end-to-end into long linear filaments (up to several micrometers) which show a tendency for increased side-by-side aggregation and formation of other higher-order structures, such as gauzes, hourglasses or rings (Figure 1.2 B-D).

Single-particle averaging methods revealed the substructure within septin complexes to be a string of six equally-sized subunits, consistent with an array of G domains (Figure 1.2 H) [60, 96]. X-ray crystallography studies of bacterially expressed human SEPT2, SEPT6 and SEPT7 revealed a nonpolar hexametric arrangement consisting of two asymmetrical heterotrimers associated head to head.
Figure 1.2: **Septin-septin interactions and septin complexes.** Electron micrographs of septin complexes formed under high salt concentration and diverse higher-order structures assembled under low ionic strength. (A) Negative-stain electron microscopy of endogenous septin heteromers prepared from human K562 cells in 0.3 M NaCl, adapted from Ref. [91]. (B) Septin filaments in 0.1 M KCl. C. and (D) Septin higher-order assemblies in 50 mM KCl, where in (C) circular bundles, and in (D) ribbon-shaped bundles of longer filaments predominate. (B-D) adapted from Ref. [54]. (E-F) Observed flexibility of human complexes around the center, adapted from Ref. [96]. (E) Class averages of the negatively stained complexes after alignment using only the left trimeric part as a reference. The trimeric part on the right adopts different orientations, indicating the high level of flexibility of the hexamer. (F) Bending from the long axis is observed in the range of 25-30°. (G) Complex purified from rat brain displaying thin strands projecting from one side, adapted from Ref. [43]. (H) The substructure of the human complex at high salt concentration is revealed after class averaging, showing a string of six equally sized subunits, adapted from Ref. [43]. (I-J) Structure and dimerization of human SEPT2. (I) Ribbon representation of the structure of SEPT2, indicating the septin-septin binding interfaces on the opposite sides of the globular G domain. (J) Ribbon representation of the part of the SEPT2 polymer with two possible dimerization interfaces labeled G- or NC-dimer, as indicated. (K) Ribbon model of the hexametric SEPT7-SEPT6-SEPT2-SEPT6-SEPT7 complex, with SEPT7 in cyan, SEPT6 in pink and SEPT2 in blue, indicating the type of nucleotide in the subunits. The boxes denote the alternating G- (solid) and NC-dimer (dashed) interfaces. The presumed orientations of the C-terminal ends predicted to form coiled coils are shown with arrows. Adapted from Ref. [113]. (L) Model of the architecture of the mammalian septin complex comprising four septin groups arranged in the sequence: SEPT3 group-SEPT7 group-SEPT6 group-SEPT2 group-SEPT2 group-SEPT6 group-SEPT7 group-SEPT7 group-SEPT3 group. The exact homologs present from each group are thought to be interchangeable and the numbers in circles denote the most prominent members.
This assembly requires two conserved interaction interfaces on the opposite sides of the G-domain: the G interface that lies across the bound nucleotide and the NC interface formed by N- and C-terminal extensions (Figure 1.2 I-K). Septin coiled-coil domains are thought to extend from the G domains perpendicular to the direction of polymer extension and play a role in stabilizing the complex [69].

Alternating G- and NC- interfaces provide a universal principle by which individual septin proteins may assemble into hetero-oligomeric units that in turn may form extended filaments. However, the investigation of crystallized recombinant SEPT2 and SEPT3 and mutational studies of overexpressed conditions of SEPT2 in cells suggest a preference of G- over NC- interface binding [53, 61, 97, 118]. In the SEPT2-SEPT6-SEPT7 complex both SEPT2 and SEPT7 are GDP-bound, and SEPT6 is GTP-und. Hence, the SEPT2-SEPT6 interface is a GDP-GTP interface, whereas the SEPT7-SEPT7 interaction contains two GDP molecules. SEPT2 forms a homodimer across the NC-dimer interce, which is not the preferred for the SEPT2 G domain alone (Figure 1.2 I,K). It is assumed that nucleotide binding and hydrolysis in specific septin subunits play a role in filament formation and disassembly; it is yet unclear why the SEPT2-SEPT2 NC-dimer interface is favored in the context of the hexamer.

The variations in the enzymatic activity of septins, as well as the importance of nucleotide binding and hydrolysis to their polymerization, are still not fully understood. The presence or absence of nucleotides in SEPT6-SEPT2 and SEPT7-SEPT7 dimers can influence their stability [97, 118]. Under specific conditions, mammalian SEPT2, SEPT7 and SEPT3 were shown to form homodimers and polymerize further into filaments in vitro (Figure 1.2 J). Though septin complex architecture appears to be highly ordered, with specific septin proteins occupying defined positions, the possibility of formation of other types of complexes is not yet ruled out.

Nevertheless, the precise and consistent order of septins within the heteromeric complexes implies some fundamental principles of assembly. Recent publications proposed that SEPT9 could extend the hexameric complex to an octamer by flanking the ends of the hexamer [52, 92]. Several mammalian septin complexes of differing composition were isolated biochemically consisting of SEPT3-
SEPT5-SEPT7 [60], SEPT4-SEPT5-SEPT8 [63] or SEPT7-SEPT9b-SEPT11 [71]. The findings employing different methods such as human septins yeast two- and three-hybrid screen, pull-down assays, density gradient centrifugation and immunofluorescent studies [52, 54, 73, 86, 91, 93] were used to investigate the properties of septin-septin interactions. It has been reported that septins within the same subgroup are able to substitute for one another structurally in vitro [54] and in vivo [91, 93]. Whether this is linked to a functional redundancy of individual septins remains unclear, however, in the SEPT5 knockout mouse, other members of the SEPT2 group seem to be upregulated [80]. From these studies it is clear that every member of the diverse septin family can be a part of septin complex and higher-order assemblies. Collectively, septins within the same subgroup are likely able to substitute for each other at the same position in the unit complex.

Physiological septin complexes are believed to be ordered heteropolymers assembled from a mixture of variable elementary septin molecules all including SEPT7, as the sole member of its group, and likely one member each of the SEPT2, SEPT3 and SEPT6 families depending on the cell types and roles of the particular higher-order septin assembly (Figure 1.2 L). This diversity could be functionally important as different septins were shown to have specific properties and interaction partners.

1.3 Mammalian septin higher-order structures in interphase cells

Little is known about physiological septin complexes and the rules that govern their higher-order assemblies. Septins are thought to exist in the cytosol exclusively as a pool of soluble heteromers [91] and in the form of insoluble higher-order structures on the plasma membrane. All expressed septin proteins contribute equally to the membrane-associated structures [91] and may shuttle between the soluble and insoluble pools.

Septins show the direct association with phosphatidylserine on the inner leaflet of cell membranes [101] and phosphatidylinositol-4-monophosphate (PIP) or -4, 5-bisphosphates (PIP2) in vitro and in mouse embryonic fibroblasts [119]. These
findings give a rise to a model of septin filament assembly where septin heteromers arrive at the plasma membrane from the cytoplasm and then form higher-order structures by annealing laterally or longitudinally. The measurements of the effective viscosity and elasticity of the plasma membrane show a dramatic decrease in membrane tethering and rigidity after depletion of septins in mammalian cells [31, 70]. Thus, septins may exist as an independent component of the membrane skeleton that influences the rigidity of the plasma membrane.

While it is clear from the above mentioned observations that septins play a role in stabilizing cell rigidity and thus must be a part of a rigid structure, it remains unclear if mammalian septins are able to self-assemble \textit{in vivo}, or if they merely associate with and strengthen other cytoskeletal polymers, such as actin. Under physiological conditions, septins are recruited to actin cytoskeleton or microtubules and it remains unclear if they require a template for the assembly.

In interphase fibroblast cells septins appear filamentous and have been shown to colocalize with long linear actin bundles, cortical arcs and subnuclear actin stress-fibers [55–57, 71, 115] (Figure 1.3 A). Early \textit{in vitro} experiments using purified mouse septin complexes showed no detectable affinity for F-actin, suggesting that an additional factor is required for recruitment of septins to actin bundles [55]. However, recent data show bundling of \textit{in vitro} assembled actin fibers by purified human SEPT9_i1 [21], drosophila or human SEPT2/6/7 complexes [64]. First, the contractile ring protein anillin was shown to efficiently engage septin complexes to bundled actin \textit{in vitro} [55]. This finding does not explain the association of septins with actin filaments in interphase cells, as anillin normally resides in the nucleus and becomes cytoplasmic only when the nuclear envelope breaks down during cell division [76]. In further studies, non-muscle myosin II (myosin II), a molecular motor component of contractile filaments, was found to colocalize with septins in resting CHO cells along actin stress-fibres [50] (Figure 1.3 B). Nevertheless, the direct interaction of actin and fly septins was shown \textit{in vitro} [64].

When actin filaments are disrupted by latrunculin B, jasplakinolide or cytochalasin D, some filamentous septins reorganize into small rings of approximately 500 nm in diameter and septins in cortical arcs remain stable (Figure 1.3 C) [52, 55, 115]. Some of the septin rings formed during drug treatment are not asso-
Figure 1.3: **Higher-order septin assemblies in the interphase cells.** (A) Association of septins and actin. Immunofluorescence images of mouse NIH3T3 fibroblasts. SEPT2 (green) is aligned as fibrous stretches along actin stress-fibers (red). Adapted from Ref. [25]. (B) Nonmuscle myosin II colocalises with septins along actin stress-fibers. Confocal images of resting hamster CHO cells transfected with GFP-myosin II (green), endogenous SEPT2 (red). (C) Septin rings formed upon actin destabilization. Mouse NIH3T3 fibroblasts treated with cytochalasin D. Immunofluorescence staining of endogenous SEPT2 (red) and phalloidin staining for F-actin (red). Septins are organized as short stubby linear filaments along residual actin bundles, and C- or O-shaped structures. Septin-positive arcs remain at the cell periphery. Adapted from Ref. [54]. (D) Septins remain associated with myosin II after F-actin depolymerization. CHO cells treated with cytochalasin D, GFP-myosin II (green), endogenous SEPT2 (red). A representative ring (dashed box) is shown in inset. (E) Association of septins with a subset of microtubules. Dog MDCK cells, expressing SEPT2-mCherry and transfected with tubulin-GFP. Arrows point to SEPT2 coated microtubules. Adapted from Ref. [7]. (B,D) Adapted from Ref. [50].
ciated with the membrane and diffuse in the cytoplasm, suggesting that they are not tightly associated with the residual cytoskeleton and may represent a default organization of septins [55]. The dissociation of septins from actin filaments is reversible: after drug washout rings disappear and septins diffuse on the membrane to later co-align with polymerized actin [55]. Noteworthy, in non-adherent human K562 cells septins appear to assemble exclusively into submembranous rings of similar diameter [90]. Furthermore, addition of recombinant hetero-oligomers of human SEPT2, SEPT6, and SEPT7 to spherical giant liposomes, with incorporated PIP and PIP2 lipids, triggers the formation of membrane tubules with diameter close to the inner diameter of circular septin assemblies [105].

While EM studies demonstrated certain flexibility in the core of septin complexes and purified septins were shown to associate in similar rings in vitro, it is still not clear if septins self-assemble in rings in mammalian cells. Essentially, the association of septins to myosin II motor remains conserved in actin-independent ring structures (Figure 1.3 D) [50].

Colocalization of septins with microtubules in interphase mammalian cells has been extensively documented by immunofluorescence microscopy [37, 49, 62, 72, 100, 103, 111, 116]. Septins decorate only a subset of microtubules that are mostly restricted to regions near the nuclear envelope, underlying Golgi sites of vesicle export and the cell periphery (Figure 1.3 E). Strikingly, septin-coated microtubules are predominately bundled and this bundling is abolished upon septin depletion [100].

In some cells, septin localization was shown to be partially sensitive to depolymerization of microtubules [72, 103]. Upon treatment with nocodazole, septins were solely localized with the actin cytoskeleton and no formation of rings was observed. Removal of nocodazole allowed for the reformation of septin-positive microtubules.

Fluorescence recovery after photobleaching experiments indicated that septin filaments are at least threefold less dynamic than microtubules [45] and exhibit twofold slower turnover than cortical actin [35]. This raises the possibility that filamentous septins may persist longer than dynamic microtubules that undergo depolymerization or catastrophe, and are more stable than average actin polymers at cell margins.
While many details of septin filament assembly and dynamics are missing, we have now a rough picture for how septins are organized on the plasma membranes of interphase cells. Here, septins control the spatial organization of membranes and the cytoskeleton in the establishment and maintenance of cellular asymmetry by blocking the lateral diffusion of membrane-associated proteins, deforming the membrane bilayer and possibly by rigidifying the cell membrane.
Chapter 2

Single-molecule super-resolution microscopy

Fluorescence microscopy is the most widely used imaging technique among biologists for the visualization of cellular constituents and is highly attractive due to short preparation times, molecular specificity and high sensitivity of signal detection. However, the resolution of conventional light microscope is intrinsically limited by diffraction to \( \sim 200 \text{ nm} \) in the lateral and \( \sim 500 \text{ nm} \) in the axial dimension, which is insufficient to resolve fine details such as relative positions of molecules in complexes. In recent years, the limit of optical resolution has been pushed down to a few nanometers with the development of several ground-breaking fluorescence super-resolution imaging techniques which have allowed to describe biological processes and resolve structures in unprecedented detail. Among them are approaches that rely on the sequential localization of individual fluorophores and the reconstruction of resulting molecular positions to a super resolution image: PALM, STORM, dSTORM. Such single-molecule localization microscopy (SMLM) approaches allow imaging at resolution compatible with macromolecular or even molecular scales (reviewed in [88]).
2.1 General concept of SMLM

Modern light microscopy relies on the fact that certain organic molecules upon excitation at an appropriate wavelength emit light in the form of fluorescence. When such molecules can be introduced to biological samples, the fluorescence microscope becomes an indispensable analytical tool for the investigation of cells and tissues. The resolving power of an optical system, a parameter often defined as the largest distance at which the image of two point-like objects can be distinguished, is fundamentally limited by the wave nature of light. The basic operating principle of a fluorescence microscope involves the illumination of the labeled specimen with an excitation source through an objective lens. In the imaging process of a lens-based microscope, light rays from a point in the object space converge to a point in the image plane. Diffraction and interference of light waves cause the point on the object to spread in the image plane instead of converging to an infinitely sharp spot. The finite-sized spot on the image plane, termed the point-spread function (PSF), determines the resolving capacity of the microscope. The only observable information about the emitting object’s structure in the focal plane lies within a diameter $d = \frac{\lambda}{2 \cdot n \cdot \sin(\alpha)}$, where $\lambda$, $\alpha$, and $n$ denote the wavelength of emitted light, the aperture half-angle of the objective, and the refractive index of the imaging medium, respectively. $NA = n \cdot \sin(\alpha)$ is known as the numerical aperture of the objective, which is in a range of 1.4-1.6 for the best commonly used oil immersion objectives. Therefore, even with optimal optics and non-scattering specimen, conventional fluorescence microscopes are unable to visualize details finer than about 200 nm in the direction perpendicular to the optical axis (lateral resolution) and 500 nm along the optical axis (axial resolution) of the objective when imaging with visible light ($\lambda \approx 550$ nm).

Although the image of a single fluorophore is manifested by the PSF, the precision of determining the fluorophore position from its image can be much higher than the limit set by diffraction. In single-molecule localization microscopy (SMLM) a so-called super-resolution image is generated by separating the fluorescence of emitters in time. Sparse subsets of fluorophores, which are isolated in at least the diffraction-limited space of the imaging instrument, are sequentially localized with sub-diffraction precision. Single-molecule positions from thou-
PART I. INTRODUCTION

sands of emitters in densely labeled sample are then used to generate a density map revealing sub-diffraction structures with a resolution in the order of \( \sim 20 \text{ nm} \) (Figure 2.1).

![Fluorescence single-molecule images

Figure 2.1: Principle of single-molecule localization based microscopy. Top row, a conventional fluorescence image acquired in at low excitation intensity. The structures below 200 nm can not be resolved. Higher irradiation intensity is applied to transfer the majority of fluorophores into a non-fluorescent OFF state and a low spot density is reached. The individual positions of the dyes can be determined. Below, image reconstructions of localized fluorescent spots. Image adapted from Ref. [110].

SMLM in biological specimens was first demonstrated by three groups independently in the form of photoactivated localization microscopy (PALM) [6], stochastic optical reconstruction (STORM) microscopy [84] and (F)PALM [42]. These methods were later followed by Ground State Depletion Individual Molecule Return (GSDIM) [26] and direct STORM (dSTORM) [40]. All of these techniques utilize the concept of reconstructing a super-resolved image by localizing the centers of the spots produced by stochastically switching single molecules. They essentially differ only in the nature of the switching process. In this work the main focus is set on methods related to dSTORM, and only those will be discussed further.
2.1.1 The photo-switching mechanism in SMLM

The concept of single-molecule localization-based imaging revolves around the ability to manipulate the photo-physical properties of fluorescent molecules. The key step of the imaging procedure is the ability to transfer the majority of fluorophores to a reversible OFF-state - a state where the fluorophore cannot absorb and emit a photon in the spectral imaging range. Spontaneous or stochastic activation by irradiation with the appropriate wavelength will leave a small, non-overlapping, fraction of fluorophores in the ON-state where they are able to absorb and emit a photon in the spectral imaging range. A very simple and powerful variation of this concept is direct stochastic optical reconstruction microscopy (dSTORM) [40] or GSDIM [26]. It was demonstrated that a number of conventional and structurally unrelated dyes including cyanines [39, 40], rhodamines [26], oxazines [112] and even fluorescent proteins [26, 114], can be directly used as photo-switches in aqueous solvents by simply adding millimolar concentrations of reducing thiols. The suffix 'direct' in dSTORM distinguishes the approach from the original implementation which requires for a proximal activator fluorophore for the photo-switching process.

dSTORM relies on the thiol-facilitated photoinduced formation of a long-lived intermediate radical anion of the fluorophore which constitutes the OFF or so-called 'dark' state (Figure 2.2 A). Here the dyes triplet state, having a comparably longer lifetime in the order of several microseconds, serves as a gateway from which it can be quenched to the dye radical anion dark state upon irradiation in the presence of millimolar concentrations of thiols. The fluorescent or ON state of the fluorophores is recovered upon oxidation by molecular oxygen. Both processes, reduction of the triplet state by thiols and oxidation of the intermediate reduced state by oxygen, are facilitated on irradiation. Excitation of the fluorescent form pumps the fluorophore into the triplet state, which is reduced by the thiolate. As a result the speed of the switching process can be controlled by the illumination intensity rather than merely by the fractional time the fluorophore spends in its dark state. Direct excitation of the radical anion near its maximum absorbance (~400 nm) has shown to efficiently facilitate the recovery of the fluorescent form (Figure 2.2 B) [19, 39].
Figure 2.2: **Reversible photo-switching mechanism of Alexa and Atto dyes in the presence of thiol**s. (A) Upon excitation from the singlet ground $^1F_0$ to the singlet excited state $^1F_1$, the fluorophore either can undergo radiative transition back to the electronic ground state or undergo inter-system crossing to the triplet state $^3F$. The triplet state may react with ambient oxygen to return to the ground state or react with the thiolate to the radical anion of the fluorophore $F^-$. The radical anion can be oxidized by oxygen to recover the singlet ground state. The lifetime of the radical anion is mainly determined by the oxygen concentration and can easily exceed several hours. (B) Absorption spectrum of Alexa Fluor 488 in the presence of 100 mM MEA before (black) and after high-intensity irradiation with 488 nm light. The main absorption at 488 nm decreases, while an additional peak appears at around 400 nm, which is the main absorption band of radical anions of most rhodamine and oxazine derivatives. The fluorescent state (black curve) can recover spontaneously or by direct excitation of the radical anion at ~400 nm (dark blue), (OFF- and ON-switching are photoinduced). Figure adapted from Ref. [110].
PART I. INTRODUCTION

In practice, continuous illumination of the sample with a light intensity above a certain threshold is used to pump the majority of the fluorophores from the ground state into the dark state. As the time molecules spend in the dark state is much longer than the fluorescence lifetime, the number of molecules in the ground state at any given time is minimized to a level that allows for the observation of well-separated single molecules. While, in this modality, an illumination with a single laser is sufficient for the stochastic switching, a moderate intensity 405 nm light is often used to increase the number molecules in the ON-state in a controlled manner. This is, for instance, necessary as fewer and fewer fluorophores become available as the imaging progresses due to photobleaching. In order to minimize the photobleaching of the dye due to oxidation by reactive oxygen species generally an enzymatic oxygen scavenging system is used [17]. Typically used thiols include β-mercaptoethanol (BME), β-mercaptopethanolamine (cystamine, MEA) or L-cysteine methyl ester (L-Cys-ME). Different dyes have shown to have different requirements for efficient switching and the right combination of dye and the imaging buffer is extremely important for the success of a dSTORM experiment.

2.1.2 Resolution in localization microscopy

The localization analysis used in SMLM methods makes use of a simple concept. While the size of an observed object is limited by the resolution of the microscope, its centroid or position can be determined with a precision in principle only limited by shot-noise, i.e. the number of detected photons N. The precision with which a single fluorophore can be localized from its diffraction limited spot scales as $N^{1/2}$. In practice, this so-called localization precision depends on a number of other factors, such as background noise, effective camera pixel size, or width of the emitter’s PSF. The localization precision can be described by the following equation (eq. 14, Ref. [107]).

$$\langle (\Delta x) \rangle = \frac{s^2 + a^2/12}{N} + \frac{4\sqrt{\pi} s^3 b^2}{aN^2}$$

(2.1)

Where $s$ is the standard deviation of the PSF, $a$ the pixel size of the camera, $N$ the number of photons detected from the fluorescent particle of interest, and $b$ the background noise. Typical sources of background noise include scattering
and autofluorescence from the sample or the microscope itself, or readout error and dark current noise from the camera. This expression was used throughout this thesis to quantify the single-molecule localization precision.

A number of different algorithms have been developed for the localization of single-molecules for SMLM, such as centroid calculation, non-linear least-squares fitting and maximum-likelihood estimation with discrete Gaussian point spread functions. Some algorithms offer the possibility of multi-emitter fitting. These are able to reliably determine single-fluorophore positions in spite of highly overlapping emission patterns from multiple emitting fluorophores within a diffraction-limited area (reviewed in Ref. [98]).

The precision with which a single-molecule can be localized represents only a theoretical lower bound on the attainable image resolution. While the classical definition of resolution requires the ability to separate objects on that distance scale, there are a number of practical limitations, so that the localization precision does not directly translate into resolution. Whether the final image accurately and fully represents the structure of interest largely depends on the efficiency and accuracy of labeling the targeted molecules.

The first problem here is the density of labeling, which is a consequence of the Nyquist theorem: in order to resolve two objects spaced at a distance $d$, the space has to be sampled in at least $d/2$ to $d/3$ intervals. This simply implies that the density of labels in the sample needs to be at least twice as high as the required resolution. A second problem arises, when the dye is not delivered into close proximity of the target structure or binds unspecifically to cellular structures, which leads to significant distortions in the reconstructed image.

Therefore, due to the nature of the technique, sample preparation for SMLM poses a significant challenge. Here, problems include the availability of highly-specific labeling probes, epitope preservation after fixation [68, 89], or epitope accessibility in dense structures [77], as well as the distance of the imaged fluorophore to the target structure (linkage-error) [82]. Traditionally, bright fluorophores are delivered to the target structure by indirect labeling with a primary target-specific antibody and a secondary antibody tagged with a bright dye (Figure 2.3 A). Here the limitations are the availability of specific primary antibodies, which stands even more acute when those antibodies should be from
different species for multi-color imaging, and secondly the relatively large size of IgG molecules, which is in the range of 10 nm. In some specific cases the latter might be overcome by directly labeling primary antibodies or Fab fragments. However, often it is not technically feasible for all primary antibodies, since they are often supplied as sera or in a buffer containing BSA for stabilization, and is generally time-consuming and expensive which makes it inflexible and only solves the problem in specific cases. For a limited number of molecules specific small binders exist, which can bring the dye into closest proximity possible to the target such as the actin-binding toxin phalloidin (Figure 2.3 D). Further efforts have been made to develop more generic small-sized binders which include DNA aptamers [23] or bicyclic peptides [10] created using in vitro selection, but these approaches have not found wide application in SMLM yet.

Other strategies to bring fluorophores close to the target structure for SMLM make use of genetically encoded labels, which comprise a broad category of tags generated in a field that has been very active due to the requirement of switchable
fluorophores for SMLM [6]. These include recombinantly expressed photoactivatable and photoconvertible fluorescent fusion proteins such as PAmCherry [102], mEos2 [67] or mMaple [65], but their superior labeling specificity through the genetic fusion with the target molecule comes at the expense of a lower localization precision due to a generally lower photon yield in comparison with organic fluorescent dyes.

Another approach makes use of the special catalytic protein fusion tags, like SNAP [29] or Halo [59] which undergo selective covalent linkages with their synthetic chemical ligands (Figure 2.3 C). This requires the generation and characterization of new constructs, whose endogenous functionality is difficult to verify, since the fusion-proteins can only be made visible via their ligands which is very complicated in live cells. Furthermore, it is still unclear to what extent the tag is functional and if quantitative labeling can be achieved.

Another method, relies on the expression of recombinant constructs labeled with fluorescent proteins, which are used as epitopes for the binding of small high-affinity alpaca antibody-fragments, called nanobodies. Camelids such as llamas and alpacas have IgG molecules composed of two heavy chains only, without light chains [36]. As a consequence, the epitope-binding hypervariable domain consists of a single amino-acid chain in a small fold (~10-15 kDa), which can easily be expressed recombinantly and has been named a nanobody. Their small size and ease of production and genetic engineering has made them promising targets for biomedical imaging and as molecular therapeutics. Several nanobodies have been developed, particularly against cytoskeletal components [109], and specific nanobodies fused to GFP have been employed for intracellular detection as chromobodies [83]. Especially nanobodies against the green fluorescent protein (GFP) have been successful reagents for in structured illumination [87] and single-molecule localization super-resolution microscopy [82].

Accurate and quantitative labeling of cellular molecules has thus become one of the bottlenecks in the race for highest resolution of target structures. Also very important are several photophysical parameters of the fluorophore used. Besides the localization precision limiting brightness (photons per switching event) there are several other properties of the dye which have an impact on the image resolution. The on/off duty cycle, which is the fractional time the fluorophore spends in
the on-state, will ultimately limit the number of fluorophores that can be resolved within a diffraction-limited area. Crucial are the number of switching cycles, as the repeated sampling of the same fluorophore will increase the precision in determining its position, and its recovery efficiency from the dark state, which presides over the proportion of fluorophores which can actually be imaged.

2.2 Multicolor SMLM methods

Although the development of SMLM super-resolution techniques has afforded a resolution in the nanometer range, their ability to resolve structures consisting of different components requires multicolor imaging. With this regard several powerful multicolor super-resolution imaging approaches have been developed and have allowed to determine the molecular architectures including cell adhesion complexes [94], neuronal synapses [15] or axons [117]. The most straightforward approach makes use of dyes with separate excitation and emission maxima and sequential recording using separate lasers and filters or simultaneous detection using a dichroic beamsplitter [22, 110]. These approaches can be easily implemented on most commercial microscope platforms. Here, commonly available fluorophores can be used, but simultaneous acquisition requires spectrally separated dyes which are compatible in terms of their switching properties in the same chemical environment. The performance of dyes used with regard to buffer-composition for simultaneous imaging is a broadly discussed problem [4, 17] and in most cases a trade-off leads to a reduced localization precision and overall resolution. A further drawback of these image modalities is the requirement for image registration. The chromatic offset in well-corrected microscope systems, being merely a fraction of an optical wavelength (<50 nm), is negligibly small in for conventional diffraction-limited imaging. However, for multicolor superresolution imaging, it will ultimately cap the compound resolution if not at least in part corrected for. In the case of sequential imaging using a dichroic beamsplitter the images need to be registered from different parts of the camera or different cameras. Here different optical paths introduce further distortions between the images. Methods to register two images with accuracies below 10 nm exist, but involve very elaborate and sophisticated calibration experiments, even more so in 3D [24, 28].
To this end, several strategies have been put forward to circumvent problems involving image registration and chromatic aberration. A very widely used approach is based on activator-reporter fluorophore pairs [4, 5]. Targets are labeled with the same carbocyanine reporter dye (e.g. Cy5, Alexa Fluor 647) coupled to a chromatically distinguishable shorter-wavelength activator chromophores (e.g. Cy2, Cy3, AF405). Excitation of the activator dye is used to drive the proximal reporter dye from the dark state into the fluorescent state. Imaging is performed with multi-laser activation-excitation cycles and the color is assigned based on the activating laser pulse. As acquisition is performed in the same channel, the approach does not require image registration and is entirely free of chromatic aberrations. However, the error introduced in the color misassignment due to false or spontaneous activation is relatively high, but can be accounted or corrected for by stochastic [4] or statistical approaches [15]. Another disadvantage entails the requirement for sophisticated labeling procedures, where two fluorophores need to be conjugated to the same label at very specific ratios. The employed labels will also determine the extent of crosstalk which will vary with the batch of the employed reagent.

An alternative way to perform multicolor SMLM is based on spectral-demixing, where two fluorescent probes are excited by the same laser line. Their partially overlapping emission is separated into two detection channels via a dichroic beamsplitter [3, 106]. As the emission spectra of the dyes are overlapping to a certain extent, both fluorophores are visible in either channel thereby producing a localization pair. The color assignment is performed based on the ratio of the signal intensities between short and long wavelength channels of the localization pair. Reconstruction of the two-color image can be done based on the localizations found in one channel as it contains the position information of both dyes. Hence all optical surfaces are identical for the two spectrally very close colors. As a result, the approach is close to free of chromatic aberrations and the two colors are inherently registered. This method, however, poses strict requirements on the choice of fluorophores regarding spectral separation and switching behavior in the same chemical environment and is difficult to extend to more than two colors.

More recently a method for multicolor imaging has been proposed which relies on the transient binding of short fluorescently labeled oligonucleotides as a vari-
ation of point accumulation for imaging in nanoscale topography (PAINT) [51]. Therein, target structures are labeled with different tags bearing different short oligonucleotides. Imager strands with sequences complementary to the DNA oligos on the structures of interest are added and read out sequentially. The method can in principle achieve very high resolutions, as the same target can be imaged repeatedly and offers the possibility of high multiplexing due to the combinatorially large number of potential oligonucleotide sequences. The method makes use of a single laser source and only a single dye and is thus free of any chromatic effects.

Another alternative to multiplexed imaging involves the sequential read-out of fluorophores which are chemically quenched after each imaging round [104]. The sample is then relabelled for the next target. However, the sample needs to be blocked and stained and washed between imaging two separate labels leading to very long measurement times. In addition, manipulations of the sample lead to mechanical distortion or shift of the sample.

2.3 Three-dimensional (3D) SMLM methods

SMLM methods for sub-diffraction 3D resolution face an additional set of challenges. The optical axis is historically the more difficult dimension with an axial FWHM of the PSF given by $dz \approx 2 \cdot \frac{\lambda}{n \cdot \sin 2\alpha} \approx 500 \, \text{nm}$, which is three times worse than the lateral resolution. Given the inherent asymmetry of a microscope’s operating principle, super-resolution along the optical axis using SMLM has required the development of a completely different approach to determining an emitter’s z-position as opposed to finding the centroid. One category of techniques is based on obtaining the z-position of a single molecule from the intrinsic shape or after engineering of the microscope’s PSF from two-dimensional images. A very widely used approach employs double-plane detection. In this so-called bi-plane FPALM (BP-FPALM) the emission is equally split by means of a non-polarizing beamsplitter between two focal planes. The two optical paths have different path lengths such that the two focal planes are axially separated by several hundreds of nanometers. The z-position of the fluorophore is determined from the widths of the PSFs from the two focal planes. In another widely used approach, a cylindri-
cal lens is placed in the emission path to introduce an astigmatism which distorts the Gaussian spot depending on the distance from the focal plane. The resulting PSF appears elliptical. And the z-position is inferred from the ratio between the two axes of the ellipse [46]. The two latter methods provide an axial resolution of \( \sim \)50-100 nm in a depth of view of several microns.

A further method employs a double-helix PSF (DH-PSF) which uses a liquid crystal spatial light modulator (SLM) to engineer the PSF into a double-helix [79]. A single fluorophore appears in the image plane as two spots at different angles depending on the axial position. The method achieves a quasi-isotropic resolution with an axial resolution of \( \sim \)20 nm over a relatively thick depth of view of 1-2 \( \mu \)m.

More recently, an isotropic 3D resolution below 20 nm with a depth of field of 3 \( \mu \)m has been shown using a self-bending PSF. The method involves splitting the emission from single fluorophores between two paths and converting it into two self-bending Airy beams using a spatial light modulator. The axial position of an emitter is inferred from the lateral displacement between the two images produced by a single molecule on the camera. Exceptionally high axial resolutions have been achieved the interferometric methods including iPALM [95] and 4Pi-GSDIM/4Pi-STORM [2]. In both methods the microscope is equipped with two objectives sandwiching the sample, and two light paths are guided to interfere at the beam splitter. The z-position of the fluorophore is calculated from the resulting interference pattern arising from the phase difference between the light collected by each objective, which depends on the distance travelled by the photons in each light path. Interferometric methods are in principle capable of providing isotropic resolution of 20 nm for samples of up to 700 nm in thickness. However, they require complex optical setups which are difficult to implement, align, calibrate and operate for the non-expert.
Part II

Method development
Chapter 3

SMLM using nanobody binders

With the recent development of single-molecule localization-based super-resolution microscopy techniques, the limit of optical resolution has been pushed down to a few nanometers, which has made it possible to image fluorescence-labeled features in unprecedented detail. SMLM methods are becoming increasingly popular to decipher the organization of sub-cellular structures. Traditionally, fluorescence microscopy is highly attractive for the ability to visualize locations and interactions of biomolecules due to relatively simple and quick sample preparation, outstanding contrast and high specificity. However, several problems that could be neglected in diffraction-limited microscopy pose significant problems for the SMLM imaging. Because existing bright fluorophores afford enough photons for a high localization accuracy of the dye, whether the final image accurately represents the structure of interest largely depends on the efficiency and accuracy of labeling the targeted molecules. Significant distortions in the reconstructed image arise if the dye is not delivered into close proximity of the target structure or binds unspecifically to cellular structures. Present labeling techniques in many cases are daunted by the number of factors that limit the range of samples suitable for investigation. These problems include the availability of highly-specific labelling probes, epitope preservation after fixation or epitope accessibility in dense

structures, as well as the linkage error of the imaged fluorophore.

In this chapter, a new approach to use small (∼10-15 kDa), high affinity nanobody binders against GFP and RFP family proteins is discussed. Many of the fluorescent proteins used today were derived from small successive modifications of only a few ancestral proteins, most prominently the green fluorescent protein from *Aquorea victoria* and the red fluorescent protein DsRed from *Discosoma sp*. The widespread usage and continuous development of such fusion proteins has led to a wealth of well-characterized constructs. The described method relies on the expression of recombinant constructs labeled with GFP and RFP derivatives, which are used as epitopes for nanobodies. Our strategy provides highly generic labeling with bright organic dyes for the application in super-resolution imaging and single-particle tracking (SPT).

### 3.1 Results and Discussion

#### 3.1.1 Labeling density and resolution in nanobody-labeled structures

As a proof-of-principle, the nanobody-based labeling approach was compared to the established methods on the example of well-defined structures in fixed tissue-culture cells. In the first set of the experiments the microtubule cytoskeleton of the rat-kangaroo PtK2 cells, stably expressing YFP-tubulin, was superresolved via dSTORM measurements using Alexa Fluor 647 (AF647) fluorophore. Three different strategies were employed to bring AF647 to the target: α-tubulin was labelled with anti-tubulin antibodies and corresponding secondary antibodies, YFP-tagged tubulin was labelled directly with anti-GFP nanobodies or indirectly with anti-GFP primary antibodies and corresponding secondary antibodies. On the reconstructed dSTORM images in all cases individual microtubules were densely labelled and their full width at half maximum (FWHM) was calculated (Figure 3.1 A-C). Compatible with microtubule diameter of 25 nm, the structures labelled with nanobodies were 26.9 nm ± 3.9 nm wide. This was significantly less than what was achieved using anti-GFP (42.7 nm ± 7.0 nm) or anti-tubulin (45.6 nm ± 5.8 nm) antibodies, suggesting that the use of nanobodies led to minimal linkage-
error (Figure 3.1 E-D).

Figure 3.1: **Nanobody labeling improves the resolution due to a minimized linkage-error.** (A-C) Reconstructed super-resolution images obtained using different labeling strategies for microtubules in fixed Ptk2 cells stably expressing YFP-tubulin. (A) Mouse anti-tubulin antibody plus secondary anti-mouse antibody labeled with AF647 (αMT). (B) anti-GFP nanobody labeled with AF647 (Nanobody). (C) Overview of a larger area and B. close-up of the area outlined by the dashed box (AF647 in green, CF680 in red). (C) mouse anti-GFP antibody plus secondary anti-mouse antibody labeled with AF647 (αGFP). (D) Intensity profiles of microtubules calculated from regions as indicated in the reconstructed super-resolution images, and the FWHM was determined by fitting with a Gaussian. (E) Measured FWHM of microtubules from at least 60 line segments and three samples per labeling method. Boxes denote median values ± quartiles (***, NB versus αMT: \( P = 3.8 \cdot 10^{-52} \); and NB versus αGFP: \( P = 2.7 \cdot 10^{-25} \); Mann-Whitney-test). Green dashed line indicates microtubule diameter.

Next, to demonstrate further that the size of the nanobody improves the resolution due to a minimized linkage-error and that the use of nanobodies supersedes the resolution obtainable using proteins directly tagged with common photoactivatable fluorescent proteins, the caveolae were imaged using different strategies. Caveolae are flask-shaped invaginations of the plasma membrane, 60-80 nm in diameter, formed by the membrane associated protein Caveolin 1 (Cav1). First, anti-Cav1 primary antibodies and AF647 tagged secondary antibodies were used to detect caveolae in rat epithelial NRK52E cells. When the dSTORM experiment
was performed, Cav1 assemblies were visualised as rings with an average diameter of \( \sim 116 \) nm, resulting from the two-dimensional projection of a hollow cup-shaped caveolae in the plane of the membrane (Figure 3.2 A). Next, Cav1-EGFP was transiently expressed in NRK52E cells and the labeling was performed with AF647-anti-GFP nanobodies. The caveolae seen in the reconstructed dSTORM image had the same morphology, but exhibited a smaller diameter of \( \sim 94 \) nm (Figure 3.2 B). Finally, PALM imaging of the caveolae in HeLa cells transiently transfected with Cav1-PAmCherry was performed. In the reconstructed images caveolae were manifest as point-clouds of \( \sim 128 \) nm in diameter and a ring shape of the structure was detectable merely in few cases (Figure 3.2 C). Although genetic tagging with a photoactivatable fluorescent proteins provides superior labeling efficiency and specificity with the smallest linkage error of the three compared labeling methods, the photon yield of PAmCherry is 4-5 times lower than AF647 resulting in a roughly two-fold lower localization precision. Taken together, it was concluded that nanobody labeling allows the resolution of finer details in SMLM measurements (Figure 3.2 D).

Lastly, we demonstrate the performance of nanobody labeling of dense multicomponent assemblies in the crowded cellular environment. The nuclear pore complex (NPC) is a symmetric, highly packed cylindrical structure of \( \sim 145 \) nm in diameter, which spans the nuclear envelope and controls the exchange between cytoplasm and nucleoplasm. Human U2OS cells stably expressing the nuclear pore protein Nup43 tagged with EGFP were used for SMLM imaging via the AF647 labeled anti-GFP nanobody. We were able to visualize ring structures of \( \sim 100 \) nm in diameter on the lower surface of the nucleus of fixed cells, which is consistent with the size of the human NPC (Figure 3.2 E). In some cases even the rotational eightfold symmetry arrangement was observed. A comparison was made with the commonly used indirect immunofluorescence method with primary anti-GFP antibody and secondary anti-mouse antibody labeled with AF647 to visualize Nup43-EGFP (Figure 3.2 F). Though the reconstructed image contained localizations that could be attributed to the NPC, it was not possible to observe the full ring structure, likely owing to steric hindrance preventing complete labeling with relatively large antibodies in the dense environment of the nuclear pore complex. Therefore, we conclude that the small size of nanobodies provides su-
Figure 3.2: **Increased labeling density and resolution in nanobody-labeled structures.**

(A) Caveolae in NRK52E cells labeled via rabbit anti-Cav1 antibody and secondary AF647 conjugated antibody. (B) Cav1-EGFP transiently expressed in NRK52E cells and labeled with AF647-anti-GFP nanobodies. (C) PALM image of Cav1-PAmCherry in HeLa cells. (D) Measured diameter of caveolae from at least 20 structures. Boxes denote median values ±SD, whiskers mark minimum and maximum values of the distribution. (E) Differences in epitope accessibility of nuclear pore complex components between nanobodies and antibodies. Images show the lower surface of the nucleus in fixed U2OS cells stably expressing Nup43-EGFP. (E) Nup43-EGFP labeled via AF647 conjugated anti-GFP nanobodies. (F) Nup43-EGFP labeled with primary anti-GFP antibodies and secondary AF647 conjugated antibody.
The sample preparation for imaging via nanobodies consists of several simple steps and can be performed in 2.5 h. This makes it very easy to perform SMLM measurements on the same day to avoid deterioration of the sample. We noted that nanobody-staining performed equally well after fixation with most common reagents such as paraformaldehyde, glutaraldehyde, or methanol, when the measures to reduce fixative specific background are taken. This is important because some structures such as actin or microtubules require specific staining conditions and not all antibodies are compatible with glutaraldehyde or methanol fixation.

The overall steps of the labeling protocol are visualized in a Diagram (Figure 3.3) and the detailed protocols for the staining procedures performed here are provided in Section 8.7.

### 3.1.2 Single-particle tracking

We next demonstrate how nanobodies can be used in the uPAINT [30] modality of SPT to study cellular membranes. Here, the protein of interest carrying an extracellular fluorescent tag is labeled with a nanobody bearing a bright and photostable dye whose spatial position can be determined with nanometer accuracy over time. In this way, the motion of transmembrane proteins can be quantitatively described on a single-molecule level.

We show here a simple and elegant way of employing nanobodies for dynamic and nanoscopic investigation of small (less than 2 μm) mushroom-shaped protrusions from the neuronal dendritic shaft termed spines. Solute and membrane-bound diffusion into the peculiar-shaped spines is widely investigated and it is still not entirely clear how spine access is mechanistically regulated for both types of influx.

In single spines of cultured hippocampal neurons we first performed fluorescence recovery after photobleaching (FRAP) experiments of membrane associated...
Figure 3.3: Protocol for nanobody labeling. Overview of the labeling protocol with anti-GFP nanobodies for SMLM imaging of subcellular structures.
CD4-mRFP (Figure 3.4 A-B). We then complemented this ensemble diffusion measurement with experiments on the single-molecule level by additionally performing SPT on the same spine in vivo tracking the same probe using Atto647N-anti-RFP nanobodies (Figure 3.4 C). A welcome advantage being that SPT also provides the geometry of the spine with less than 15 nm resolution which is required to apply mathematical diffusion models. The combination of SPT and FRAP can help to overcome artifacts inherent to each of the methods and to provide a more accurate description of membrane diffusion.

Figure 3.4: **Single-particle tracking and fluorescence recovery after photobleaching.** (A-C) Combined fluorescence recovery after photobleaching (FRAP) technique and single-particle tracking measurement on a dendritic spine of a cultured hippocampal neuron expressing CD4-mRFP. (A) Individual frames of an image sequence recorded during a FRAP experiment. (B) Fluorescence recovery curves obtained from five consecutive FRAP experiments in color and their mean in black. (C) Single-molecule tracks obtained during SPT via Atto647N-anti-RFP nanobodies on the same dendritic spine as in (A).

### 3.2 Conclusions

In conclusion, nanobody-mediated labeling provides a simple and generic approach to label virtually any of the widely available GFP- and RFP-derived fusion constructs for advanced single-molecule imaging applications including single-particle tracking and single-molecule localization microscopy. The advantages include very precise and consistent labeling as well as superior accessibility in complex and dense samples due to the high affinity and small size of the nanobodies. For SMLM imaging applications, sample preparation is very robust and in-

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volves only a few simple steps. Images can be acquired as early as two hours after fixation. In addition, nanobodies do not show an increased off rate in the presence of reducing agents necessary for photoswitching, which can be the case for antibodies or phalloidin conjugates. Hence, the same sample can be imaged for prolonged periods and multiple image series can be generated without any loss of localization density and image quality. Nanobodies are also insensitive to the choice of fixation reagent expanding the number of specimens which can be studied.

Finally, we demonstrate the application of nanobodies for SPT measurements in combination with the ensemble technique fluorescence recovery after photobleaching. The latter provides better insights into the global and local aspects of protein motility in the plasma membrane while SPT with nanobodies provides better access to spatially confined membrane regions (e.g. the basal membrane or synapses). Conveniently, after the FRAP experiments the same probe is still available for SPT measurements and results can be directly compared. The monovalency of the nanobody assures the absence of crosslinking.
Chapter 4

GFP- and RFP-based two-color SMLM by spectral-demixing

Precise determination of the spatial relationship between different cellular constituents can be crucial to the understanding of their biological function. Despite the development of several advanced methods towards this goal [5, 94], multicolor superresolution fluorescence imaging still remains a difficult task because of a number of technical issues. If not properly addressed, these problems can compromise the resolution or color-assignment between channels to the extent that there is no additional benefit of performing multicolor imaging. This is one of the main reasons that have prevented the broader use of multicolor SMLM methods. While the most prominent techniques have been discussed in more detail in Section 2.2 of the Introduction, the most important problems concern buffer-compatibility for simultaneous imaging [4, 17], color-crosstalk [4, 15] and image registration [1, 81]. Other techniques have been able to largely circumvent these problems by using multiplexed single-color imaging. They, however, require cumbersome or time-consuming manipulations to the sample which can easily introduce mechanical distortions or shift of the sample and add further error.

With this regard, this chapter includes the development of a simple and highly generic strategy for two-color dSTORM of any combination of GFP- and RFP-tagged proteins with the use of only two nanobodies. The method combines

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1The results of this chapter contributed to the publication: E. Platonova, C.M. Winterflood, H. Ewers. ACS Chem. Biol. DOI: 10.1021/acschembio.5b00046 (2015).
the versatility and superiority of nanobody-labeling which have been discussed in detail in Chapter 3 with advantages of a spectral-demixing dSTORM imaging modality. A number of important issues related to two-color imaging are addressed by the newly developed method. High-quality two-color imaging, with a resolution down to the limit typically achieved with a single color, was performed on a number of nanoscopic structures and its application is shown towards the elucidation of septin-filament assembly.

4.1 Results and Discussion

4.1.1 Establishment of dual color dSTORM

The previously reported spectral-demixing dSTORM technique was combined with the use of the two commercially available nanobodies against GFP and dsRed and their variants. The nanobodies were labeled with Alexa Fluor 647 (AF647) and CF680, respectively for dual color staining. These dyes are highly performant in terms of brightness and duty-cycle in the same conventional blinking buffer, a prerequisite for successful simultaneous imaging.

Both dyes are imaged in parallel and can be efficiently excited by the same 643 nm laser line. Their highly overlapping emission is spectrally separated by use of long-pass dichroic beamsplitter and imaged onto two separate parts of the camera chip (a detailed description of the setup can be found in Section 8.1). Due to the spectral overlap of AF647 and CF680, their emission is not quantitatively separated by the dichroic and the two dyes are detected in both short and long wavelength channels. As a result, a single emitting AF647 or CF680 molecule produces two fluorescent spots, one in each channel, which can be identified as a pair based on the geometry of the split image view (Figure 4.1). The distribution of fluorescence between the two channels is different for the two dyes, allowing for their identification based on the ratio metric intensities between the channels.

In this particular case we used a 690 nm long-pass dichroic which splits the emission roughly into 45%/55% for AF647 and 80%/20% for CF680 between the long and short wavelength channels. The assignment to either dye of each localization pair is based on the normalized intensity ratio $r = \frac{(I_s - I_l)}{(I_s + I_l)}$, where $I_s$ and $I_l$ represent the intensity in the short and long wavelength channels, respectively.
PART II. METHOD DEVELOPMENT

Figure 4.1: **Two-color imaging mode.** (A) Emission spectra of AF647 (red) and CF680 (green), the transmission of the dichroic beamsplitter (black), the band of transmitted fluorescence by the emission filter (gray box), and the 643 nm laser line (blue). (B) Sample frame showing the split-channel view where AF647 and CF680 can be distinguished by their relative fluorescence intensity between short and long wavelength (WL) channel. The dashed line separates the channels. The final two-color super-resolution image is reconstructed from the localizations in the long WL channel.

where $I_s, I_l$ are the fluorescence intensities of the localization pair in short and long wavelength channels, and is approximately -0.1 for AF647 and 0.6 for CF680, as illustrated in Figure 4.1 B. Reconstruction of the final two-color image is based on the localizations found only in one channel. In this case, the long wavelength channel is the most favorable for CF680 due to the detected 80% of emission signal. All optical surfaces are therefore identical for the two spectrally very close colors. As such, the approach is free of field distortions and close to free of chromatic aberration and the two colors are inherently registered.

First, the performance of the two-color imaging modality was examined in terms of resolution and crosstalk using conventional antibodies as labels. The method was tested on antibody-stained microtubules. These have become a generally accepted standard for benchmarking super-resolution methods. In a first experiment, to examine the extent of residual chromatic aberration due to the $\sim 30$ nm spectral shift between AF647 and CF680, microtubules were stained in fixed CV1 cells with a primary anti-tubulin antibody and 1:1 mixture of AF647 and CF680 labeled secondary antibodies. The resulting dual color image is shown in Figure 4.2. A very high density of localizations was obtained in both colors.
due to the excellent performance of the two dyes. Ten short microtubules segments were taken throughout the field of view and the average offset between the two colors was determined to be as low as 2.2 nm (Figure 4.2 C).

Figure 4.2: Two-color super-resolution imaging of microtubules stained both with AF647 and CF680-labeled secondaries. (A) Overview of a larger area and (B) close-up of the area outlined by the dashed box (AF647 in green, CF680 in red). (C) The averaged transversal profile of ten ∼200-500 nm long segments chosen throughout the field of view fitted to a Gaussian, where Δx = 2.2 nm corresponds to the lateral shift between the two fits.

This experiment could not give direct access a) to the extent of crosstalk, since by default all localizations lay on the same microtubules and with that it could also not give direct access b) to the resolution in the single channels. Therefore, two separate experiment were prepared in which microtubules were stained either with AF647 or CF680. These were then imaged using the spectral-demixing optical configuration. From the two acquisitions semiartificial data was created by summing sequential frames pairwise, one frame from the experiment with AF647 and one from the experiment with CF680. The localization and pairing analysis was then run on the data. The result in Figure 4.3 shows that the two dyes can be clearly separated according to the normalized intensity ratio oft the localization-pair. The two-color super-resolution image shows that microtubules from the measurement with AF647 are essentially entirely red, while those with CF680 are entirely green, verifying that they have been rightly assigned to a single channel each. This is also the case where microtubules labeled with different dyes are much closer than the diffraction limit.

Image reconstruction was based on the spatial information from the long wave-
Figure 4.3: Two-color reconstructed image generated from two separate measurements of microtubules labeled with either AF647 (green) or CF680 (red). (A) Reconstructed image generated from two separate measurements. The camera frames were merged before localization analysis. (B) Averaged transversal profile of the microtubule segments outlined by the white box. The double peaks are 37 nm and 33 nm apart for AF647 and CF680, respectively. (C) Histogram of the normalized intensity ratio $r$ for the measurement shown in (A) The dashed line delimits the assignment of the localization pair to AF647 ($r < 0.15$) or CF680 ($r > 0.15$). (D) Single-molecule localization statistics for the two fluorophores.
PART II. METHOD DEVELOPMENT

length channel only, whereas the short wavelength served for the color assignment. It was therefore possible to choose two separate photon-count cutoffs for single-molecule localization in the two emission channels. A higher photon-count cutoff was chosen for the long-pass channel while a lower photon cutoff (typically half) was used for the short wavelength channel. By this, unpaired localizations could be kept with only negligible probability of false assignment. Unpaired localizations arise from dimmer CF680 blinking events that do not exceed the fluorescence intensity cutoff in the short-wavelength channel but nevertheless afford high-precision localizations for the final image reconstruction. These unpaired localizations could not be accounted in the histogram shown in Figure 4.3, but did not introduce any considerable crosstalk. This would be most likely from the AF647 into the CF680 channel, as unpaired localizations were automatically assigned to CF680. For both colors the method allowed for the detection of the double-line profile with \( \sim 35 \text{ nm} \) spacing, of the microtubules, which is indicative of an exceptionally high resolution. The projection of the 25 nm-diameter microtubule cylinder broadened by primary and secondary antibody labeling has shown to produce this effect [17]. An average localization precision below 6 nm could be achieved in both channels. It is noteworthy, that a high localization precision was achieved despite the summing of background from two measurements.

Finally, to demonstrate the quality of spectral separation of the method, structures of well-known and distinctive shapes were visualised. Reconstructed super-resolution images showed well-separated microtubules and caveolae (Figure 4.4).

4.1.2 GFP and RFP-based dual color SMLM

In the previous section the principle performance capability of the imaging modality was established to now proceed in implementing the nanobody-based two-color labeling scheme. At first, a positive control for colocalization in the range of few tens of nanometers was used consisting of tubulin-EGFP and tubulin-RFP coexpressed in U2OS cells. The overexpressing cells were fixed and stained with CF680-labeled anti-GFP and AF647-labeled anti-RFP nanobodies. The two-color super-resolution image displayed densely labeled microtubules, and from a Gaussian fit of a microtubule segment we determined a FWHM of \( \sim 30 \text{ nm} \) in
Figure 4.4: Two-color super-resolution image of microtubules and caveolae in fixed NRK52E cells. Microtubules are labelled with anti-tubulin primary antibodies, caveolae with primary anti-Cav1 antibodies and AF647 (green) or CF680 (red) secondary antibodies.
either channel (Figure 4.5 B). This is closer to the actual microtubule diameter of ~25 nm compared to experiments with antibodies, where diameters generally range between 40 and 50 nm, which is also shown in Section 4.1.1. As described in a previous work [82] and discussed in Section 4.1.1, this is a result of the reduced linkage error when using the substantially smaller nanobodies. Gaussian fitting of a single microtubule stretch in both channels displayed an offset of only 4.3 nm between the two colors (Figure 4.5 B). Microtubules represent a quasi-one-dimensional system of limited significance and next it was tested whether the nanobody-based approach can be used to detect colocalization of two different molecules within a confined nanoscopic structure. Caveolae were ideally suited, being 60-80 nm-diameter flask-shaped invaginations of the plasma membrane. The GFP and mCherry-fusions of the caveolar scaffolding protein Cav1 were co-expressed in human U2OS cells. Two-color SMLM imaging showed excellent colocalization of the two fusion proteins on a scale of few tens of nanometers within caveolae (Figure 4.5 C). In addition, the hollow shape of the caveolae was resolved in both colors, which is manifested as two peaks less than 50 nm apart in the lateral profile (Figure 4.5 D).

Next, we challenged our method to visualize two closely spaced, but morphologically different components of internalizing caveolae. Dynamin2 is a GTPase that was shown to associate with caveolae and operates as a fission collar around the neck of mature caveolae initiating their internalization from the plasma membrane to form cytosolic vesicles. The dominant negative form of dynamin2, Dyn2K44A, a mutant that cannot bind GTP, inhibits caveolar fission and arrests mature caveolae on the plasma membrane with Dyn2K44A accumulated around not completely constricted necks. When Cav1-mCherry and Dyn2K44A-EGFP were transiently co-expressed in U2OS cells, and then labeled with AF647-anti-GFP and CF680-anti-RFP nanobodies, we could clearly resolve by SMLM imaging the association of Dyn2K44A-EGFP localizations with Cav1-mCherry-positive structures and found the Dyn2K44A-EGFP staining to appear as a discrete spot in the center of the caveolae (Figure 4.6 C). Consequently, in the lateral profile of a single caveolae, Dyn2K44A-EGFP staining appears as a single peak centered between two peaks for Cav1-mCherry (Figure 4.6 D).

The ultimate goal of any multicolor SMLM technique is to reliably resolve
Figure 4.5: **Two-color imaging of α-tubulin and caveolin in U2OS cells.** (A) Image of α-tubulin double transfectants (tubulin-EGFP/tubulin-RFP) and (B) transversal profile for both colors (open circles) of the microtubule segment outlined by the box in (A). The FWHM was determined by Gaussian fitting (solid lines; (B)), and Δx = 4.3 nm corresponds to the lateral shift between the two fits. (C) Image of caveolin double transfectants (Cav1-EGFP/Cav1-mCherry) and (D) profile along the box shown in (C). The lateral profile shows a double-peak arising from the cup-shape of caveolae.
Figure 4.6: **Two-color imaging of caveolin and Dyn2K44A in U2OS cells.** (A) Two color images of individual caveolae on the basal membrane of fixed U2OS cells containing Cav1-mCherry (red) and Cav1-EGFP (green) and a schematic view of the relative localization of EGFP (green) and mCherry (red) tagged Cav1 molecules on the caveolar membrane. (B) Intensity profiles of Cav1-mCherry (red) and Cav1-EGFP along the box shown in (A). The lateral profile shows a double peak owed to the cup-shape of caveolae with similar radial distances from the center. (C) Two color images of individual caveolae containing Cav1-mCherry (green) and Dyn2K44A-EGFP (red) and a schematic view of the relative localization of Cav1-mCherry (green) on the caveolar membrane and Dyn2K44A-EGFP (red) accumulated around the caveolar neck. (D) Intensity profiles of Cav1-mCherry (green) and Dyn2K44A-EGFP (red) along the box in (C) showing as a single peak centered inside the two peaks of Cav1.
different molecules on the scale of the resolution in principle obtainable in a single color. Also the understanding of septins could greatly benefit from nanometric two-color resolution as it offers the ability to determine the spatial relationship between all the different subunits and to find the rules which govern their assembly. Septins are discussed in detail in Chapter 1, but according to the current model, their filaments are composed of a mixture of hexameric and octameric complexes. In the octamer the 25 nm long hexamer arranged as SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7 is flanked by SEPT9 on both ends. In order to visualize the ultrastructure of septin filaments by two-color super-resolution we transiently co-expressed GFP and mCherry fusion constructs of two different septin monomers in U2OS cells: SEPT2, which occupies the central positions and SEPT7, which occupies the terminal positions in the hexameric complex. Two color images of fixed cells exhibited a strikingly periodic pattern of localizations for both proteins on the same filamentous structure (Figure 4.7 A,B).

The two-color super-resolution images of septin filaments were analysed in terms of the spatial cross-correlation (see 8.4 in Materials and Methods for data analysis). The cross-correlation between SEPT2-EGFP and SEPT7-mCherry signals, averaged over 120 filaments (~60 μm total filament length), showed a periodicity with groups of peaks centered at lag distances of approximately 8, 30, 60, and 85 nm, corresponding to a lag spacing of 20-30 nm (Figure 4.7 C,D). Indeed, the shortest distance between SEPT7 and SEPT2 would be on the order of 8 nm. Taken together, the conclusion based on the measurements is (i) two different proteins within septin filaments were successfully localized within a few tens of nanometers and (ii) SEPT2 and SEPT7 exhibit a periodic organization therein.

4.2 Conclusions

In conclusion the advancement and implementation of a very powerful two-color method was achieved. On the technical side, the approach overcomes a number of difficulties common to many two-color SMLM techniques. These issues include complexity and cost of the experimental setup, color cross-talk, chromatic aberration, color registration, dye performance and buffer compatibility. Further, the method was combined with a highly versatile approach for SMLM-labeling.
Figure 4.7: **Two-color imaging of septin filaments in double transfected U2OS cells.** (A) Image of elongated septin structures on a larger area of the plasma membrane and (B) a series of images of individual septin filaments (SEPT2-EGFP in green, SEPT7-mCherry in red) and SEPT7-mCherry (red) (C) Spatial cross-correlation analysis of SEPT7 and SEPT2 (SEPT7 × SEPT2) averaged for 120 filaments from four different cells. (D) Cross-correlation of three random subsets of 40 filaments taken from the 120 filaments analyzed in (C).
in two colors which eliminates problems concerning availability and quality of
antibodies, linkage-error, and antigen accessibility. The presented method can
provide high-quality two-color super-resolution with virtually any combination
of the widely available GFP and RFP-derived fusion constructs with the use of
a single pair of nanobodies. Two-color imaging was shown with a compound
resolution close the limit routinely achieved in one color and was able to resolve
different proteins within macromolecular complexes well enough to afford insight
into their nanoscopic organization.
Chapter 5

3D dual color\textsuperscript{1}

In biological contexts it is often necessary to perform multicolor imaging while also having access to the third dimension. This section further introduces a novel dual color 3D imaging technique, which is a very simple but powerful extension of two-color spectral-demixing with bi-plane imaging for additional sub-diffraction axial resolution. The imaging approach eliminates most problems inherent to current two-color SMLM strategies discussed in Chapter 4. The performance of the method was analyzed a compound two-color 3D resolution is demonstrated close to what is routinely only achieved in single-color.

5.1 Results and Discussion

In conventional bi-plane the emission is equally split by means of a non-polarizing beamsplitter between two focal planes and imaged onto two separate parts of the camera chip. The two optical paths have different lengths so that the two focal planes are axially separated by \(\sim 1 \mu\text{m}\). The z-position is determined from the width of the two spots produced by the emitter.

The same dichroic used for two-color imaging by spectral-demixing can also be used to introduce an axial separation between the focal planes. While in conventional bi-plane imaging a formidable 50\% of an emitter’s signal is sacrificed,

\textsuperscript{1}The results of this chapter contributed to the publication: C.M. Winterflood, E. Platonova, D. Albrecht, H. Ewers. Biophys. J. DOI:10.1016/j.bpj.2015.05.026 (2015).
leading to a loss in lateral resolution, by this approach it can be exploited to spectrally identify the fluorophore for multicolor imaging. We again used the well-performing AF647 and CF680 combination of dyes. In brief, single AF647 and CF680 molecules are distinguishable by their relative intensities while their z-position is determined from the widths of their PSFs between the two channels. The concept is illustrated in Figure 5.1 B. Where in conventional bi-plane the emission is ideally split 50/50, this is not possible for both fluorophores simultaneously, and also undesirable for spectral-demixing, as the two dyes would be indistinguishable. However, there is a tradeoff between the localization precision in xy and z, so that the loss in axial localization precision of the fluorophore with uneven intensities is compensated with an increased lateral resolution. A mostly undesirable property of dichroic beamsplitters, the dependence of the spectral edge on the angle of incidence, was taken advantage of. It was possible to use the dichroic employed in Chapter 4, as depending on the incidence angle, the edge of the dichroic beamsplitter could be shifted by ±10 nm from the 690 nm nominal edge for a 45° incidence angle. By adjusting the angle to ~50° from the optical axis it was possible to shift the edge to ~700 nm to obtain the spectrum shown in Figure 5.1 A.

This resulted in a ~50/50 separation for the less bright CF680 and ~75/25 separation for the brighter AF647. Thereby, very similar intensities were obtained for CF680 and AF647 in the long wavelength channel. The z-calibration curve for bi-plane imaging using the dichroic beamsplitter is shown in Figure 5.2.

Again, we examined the performance of the two-color 3D imaging method in terms of resolution and crosstalk using microtubules. Microtubules in fixed U2OS cells were stained using a primary anti-tubulin antibody and a 1:1 mixture of AF647 and CF680-labeled secondary antibodies. Figure 5.3 shows the results of the dual color 3D imaging.

Despite having expanded PSFs, a densely labeled and by virtue of the experiment highly overlapping structure, the two dyes displayed well-separable peaks in the histogram of normalized intensity ratios (Figure 5.3 C). By choosing stringent cutoffs for the dye-assignment, we could keep the crosstalk in either channel well below 1%. As shown in Figure 5.3 D the two colors are per se fully registered. From a number of short microtubule segments throughout the field of view and at
PART II. METHOD DEVELOPMENT

Figure 5.1: **Two-color 3D imaging mode.** (A) Emission spectra of AF647 (green) and CF680 (red), the transmission of the dichroic beamsplitter (black), the band of transmitted fluorescence by the emission filter (gray box), and the 643 nm laser line (blue). It is to note that the edge of the long-pass dichroic is different compared to Figure 4.1 to suit bi-plane imaging. (B) Sample frame showing the split-channel view where AF647 and CF680 can be distinguished by their relative fluorescence intensity between short and long wavelength (WL) channels. The z-position is determined from the two widths of their point-spread functions. The final two-color 3D super-resolution image is reconstructed from the localizations in the short WL channel.

Figure 5.2: **Z-Calibration curve for bi-plane imaging using the dichroic beamsplitter.** A z-stack of 200 nm far-red fluorescent beads (660/680 fluospheres, Life Technologies) with 10 nm steps was recorded using the piezosystem at the microscope objective. The beads were embedded in a 1.5% (w/v) agarose gel, which has a refractive index close to water. Plotted is the difference between the squares of the width (standard deviation) of the PSFs in the long ($\sigma_2$) and short ($\sigma_1$) wavelength channel, which is in good approximation linear in $z$ over a distance of $\sim 1 \mu$m.
Figure 5.3: **Two-color 3D super-resolution measurement of microtubules using a mixture of AF647 and CF680-labeled antibodies.** (A) Image reconstructed from AF647 localizations and (B) from CF680 localizations. (C) The corresponding distribution of intensity ratios $r$ fitted to two Gaussians (red, green dashed lines). The red, green vertical lines delimit the assignment of a localization pair to either AF647 ($r < -0.4$), or CF680 ($r > -0.23$). (D) Two-color representation of a larger area in (A,B). (E) Histogram (2.5 nm binsize) of the average lateral and F axial distances of the microtubule segments outlined in (G). The dashed lines in red and green are Gaussian fits with a FWHM of 51 nm/57 nm and 79 nm/86 nm for AF647 and CF680, respectively. $\Delta x$ and $\Delta z$ are the offset between the fits of the two colors. (G) 3D image reconstructed from both AF647 and CF680 localizations.
different z-positions (shown in Figure 5.3 G) a lateral and axial offset of less than 9 nm between the two channels was determined (Figures 5.3 E,F). For the same dataset the average lateral and axial FWHM of the microtubules was 51 nm/57 nm and 79 nm/86 nm for AF647 and CF680, respectively. This is very close to the performance typically achieved in single-color experiments. The xy localization statistics for the experiment are provided in Figure 5.4.

![Localization statistics for AF647 and CF680 for the data shown in Figure 5.3.](image)

**Figure 5.4:** Localization statistics for AF647 and CF680 for the data shown in Figure 5.3. Only localizations with a localization accuracy below 30 nm were considered. The localizations were assigned according to the cutoffs shown in Figure 5.3 C.

### 5.2 Conclusions

In conclusion, a simple and robust method for high-quality simultaneous two-color/3D imaging was developed. The straightforward approach addresses a multitude of important problems. The most important of which are effects from chromatic aberration and requirement for color registration. Even in well-corrected microscope systems lateral and axial chromatic offsets can easily amount to several tens of nanometers and require for post-acquisition color-registration. Though possible, registration accuracies below 10 nm for 3D imaging involve extremely elaborate, technically sophisticated and time-consuming calibration measurements.
which, moreover, need to be repeated for every different state of the microscope [28].
The developed method provides 3D images with colors inherently registered within
less than 10 nm. The method was successfully used for imaging of various nanoscopic
structures in hippocampal neurons, including synapsis and the axonal initial seg-
ment. It has, for instance, allowed to precisely determine the previously unknown
relative organization of important scaffolding proteins in the axon initial segment
(Winterflood et al, under review).
Part III

Single-molecule microscopy of septins
Chapter 6

Application of SMLM methods to study the septin cytoskeleton in mammalian cells

6.1 Results and Discussion

Mammalian septins are a large family of GTP-binding proteins considered to be a component of the cytoskeleton due to their tendency to assemble into filamentous structures. Septin polymerization is considered essential for their function at cell cortices where they serve as scaffolds and lateral diffusion barriers.

Like microtubules and actin filaments, at the molecular level, septins assemble into one-dimensional polymers from globular proteins. In vitro it was shown that both recombinant and purified septins assemble into rod-like complexes as palindromic hetero-hexamers of \( \sim 27 \) nm length or octamers of \( \sim 35 \) nm length that do not polymerize further under high salt conditions. Later it was confirmed, that in the cytosol of live cells all septins exist as a mixture of already polymerized hexamers and octamers, but not as monomers. The assembly of complexes is considered as a first step towards filament formation.

Under lowered ionic strength and in vitro, septin complexes polymerize further into long, frequently pairwise-aligned filaments that form bundles, rings or gauzes. In live or fixed interphase cells, however, septin assemblies at the plasma
membrane are said to be exclusively associated with actin or the microtubule cytoskeleton in the literature. The organization of these cortical septin assemblies is not well understood. Do observed \textit{in vitro} linear filaments formed by end-to-end associated complexes reflect how septins are organized within functional assemblies \textit{in vivo}? Are complexes in functional filaments highly ordered or assemble along templates, such as actin, in a semi-organized manner?

In this chapter, the molecular basis of septin assembly in higher-order structures is addressed. The resolution below 10 nm, achievable by SMLM methods, is theoretically sufficient to visualize individual septin complexes along functional filaments in cells. When the central dimers of septin complexes incorporated into higher-order structures are localized with such precision, their pattern can provide the necessary information on the structural order.

6.1.1 Morphology of the septin cytoskeleton in interphase mammalian cells

In order to study the molecular architecture of cortical septin assemblies in mammalian cells, a suitable population of filaments was identified. First, the cortical septin cytoskeleton of two different cell types, fibroblast (NRK49F) and epithelial (NRK52E) rat kidney cells, was investigated using confocal microscopy. Each SEPT2 molecule in these employed genome-edited cell lines, generated previously in our laboratory, bears a fluorescent EGFP tag, which allows for the quantitative investigation of endogenous protein localization without inducing undesirable effects of overexpression, or the need for immunostaining.

Fibroblast cells exhibited long, filamentous septin structures, resembling linear actin bundles, cortical arcs and sub-nuclear actin stress-fibers (Figure 6.1 A). Between parallel grids of wider and brighter septin filaments, spanning across almost the entire length of the basal membrane, was a mesh of finer and dimmer filaments. This suggests that a significant fraction of septin assemblies were closely associated or bundled. In epithelial cells, cortical septin organization was dramatically different: the basal membrane was covered with very short (almost diffraction-limited) curved filaments (Figure 6.1 B). The density of these filaments was higher under the nucleus and their brightness and length appeared relatively
Figure 6.1: **Cortical septins in interphase cells.** Confocal images of the EGFP-SEPT2 on the basal membrane of genome-edited (A) NRK49F fibroblasts, and (B) NRK52E epithelial cells.

Septins were further labeled with AF647 for the SMLM investigation. Super resolved septin filaments in fibroblast cells exhibited thin chains of clouds of single-molecule localizations, spanning long distances, crossing each-other and bundling into thicker assemblies, and a small number of short separate individual chains (Figure 6.2 A). dSTORM septin localizations in the basal membrane of epithelial cells exhibited a large number of short elongated series arranged in the filaments resembling the ones identified in the confocal images (Figure 6.2 B). In both cases the spacing between localizations appeared somewhat periodic. As in the further experiments the goal was to describe the periodicities of septin localizations along the filaments, the observed bundling and variety in size and morphology of the filaments, observed in fibroblast cells, poses a complication. Due to the homogeneity of the filaments, the epithelial NRK52E cells were chosen for the further investigation.
Figure 6.2: **dSTORM images of septins in interphase cells.** The basal membrane of genome-edited (A) fibroblasts and (B) epithelial cells.
6.1.2 Relative organization of SEPT2 molecules along filaments

We investigate here whether septin complexes assemble in an end-to-end fashion in cells. When the central dimers of septin complexes incorporated into higher-order structures are localized with precision below 10 nm, their pattern can provide the necessary information on the structural order. To determine whether SEPT2-dimers occur in regular intervals along the higher-order septin assemblies, the spacing of their superresolution localizations along the previously specified filaments was examined. The developed nanobody labeling technique (Chapter 3) in combination with the genome-edited NRK52E cell-line was used to visualize septin complexes along functional filaments in fixed cells. As every expressed SEPT2 must be tagged with EGFP in these cells, the small high-affinity anti-GFP nanobodies provide the necessary tool for delivering bright organic fluorophores in the crowded environment of the cortical cytoskeleton and offer maximum labeling efficiency of the central domain of septin complexes.

The dSTORM measurements were performed under TIRF illumination to make sure that only the flat parts of the basal membrane close to the coverslips are visualized. Images were obtained with a resolution of less than 10 nm and septin filaments, as expected, were manifested as thin curvy chains of seemingly periodic localizations.

A very simple and established method in image processing, spatial autocorrelation, was used for detecting a pattern of repetition of SEPT2 within the filaments. First, a larger number of filaments were manually cropped from the superresolution images in order to yield localization data arising solely from intact filaments and to omit background localizations as well as localizations of single septin complexes on the membrane. The selected filaments, on the order of 50-200 per cell, were around 300 nm to 500 nm long and consisted predominantly of distinct clusters of localizations. In this case only filaments with a minimum of 4 clusters were selected. The filaments were ‘straightened’ and the localization data was converted to pixel-intensities. The pixels were averaged along the transverse filament axis to obtain a vector on which the autocorrelation function was computed (see Section 8.4 for the detailed procedure).

The autocorrelation graph for the 1D septin profile averaged for 202 selected
filaments is shown in Figure 6.3. It manifests a general periodicity of \( \sim 50 \) nm, which is significantly larger than the \( \sim 27 \) or 35 nm expected according to the current model for end-to-end assembled hexamers, octamers, or a mixture thereof. The observed larger distance in this analysis might arise due to incomplete labeling, for example, if only a fraction of the endogenous septins were labeled with nanobodies. However, this is in contradiction with the fact that (i) for incomplete labeling, being random, there would be no preference towards the observation of longer correlation distances compared to shorter ones and (ii) individual filaments did show a high intrinsic periodicity albeit consisting of only 4-10 distinct clusters of localizations. The periodicities observed were very variable between different filaments. Some filaments showed periodicities of 30-40 nm and 70-80 nm, while the majority had a periodicity of 50-60 nm (Figure 6.4). The larger periodicities do not fit the hypothesis of insufficient labeling, given the very small probability for the observation of equidistant clusters of localizations along the filaments.

To test if the analysis was biased during the selection of filaments, the radial distribution function was employed. It describes how the density of localizations
Figure 6.4: Variability of SEPT2 periodicity in septin filaments observed by SMLM. Shown are super-resolution images, three each, of filamentous septin structures on the membrane displaying a different periodicity with their corresponding autocorrelation function shown below. Filaments with a 30-40 nm (top row), 50-60 nm (middle row) and 70-80 nm (bottom row) periodicity. The filaments were straightened and their fluorescence intensity was averaged along the transverse filament axis for autocorrelation.
varies as a function of distance from a reference point and was computed for the entire SMLM image of septins on the membrane to obtain their average radial distribution. The computational cost was reduced by using pixel intensities (see Section 8.5 for the detailed procedure). The radial distribution was computed for two separate SMLM images. It shows a first high peak at $\sim 20$ nm, which is the distribution of localizations from the same single-molecule, and a very pronounced nearest-neighbor peak at $\sim 47$ nm, which is close to what was observed using autocorrelation analysis. Interestingly, a less distinct peak at $\sim 33$ nm and $\sim 70$ nm was observed, which is in excellent agreement with the findings of different frequencies from the autocorrelation analysis shown in Figure 6.3.

6.1.3 Two-color SMLM of actin and septin filaments

The observed variety in the periodicities of SEPT2 molecules along filaments may arise from the association of the filaments to different cellular elements, e.g. the plasma membrane or F-actin. As the next step, we tested the association of the observed filaments with the actin cytoskeleton. Confocal images of EGFP-SEPT2 and F-actin labelled with fluorescent phalloidin provide little insight about their interaction (Figure 6.5 A) as the fine actin filaments cannot be resolved by conventional fluorescence microscopy. A more detailed picture was obtained when we employed the developed two-color SMLM technique (Chapter 4) in order to visualize the interaction of endogenous septin and actin on the membrane of fixed NRK52E cells with $\sim 10$ nm resolution. We were able to detect fine actin meshes together with septin assemblies underlying the basal membrane and there was no evidence for actin-independent septin structures. The most obvious colocalization was detected around actin nodes or asters, but also along thin long actin cables (Figure 6.5 B).

6.1.4 Two-color SMLM of functional septin-septin interactions

To get further insight on the orientation of septin complexes in the filaments, two different septin homologs: SEPT2 in the center and SEPT7, flanking the ends of hexameric complex, were visualized simultaneously in septin assemblies of fixed NRK52E cells. Endogenous SEPT7 was labeled with primary and secondary
Figure 6.5: **Septin association with actin in NRK52E cells.** (A) Confocal and (B) superresolution images of endogenous EGFP-SEPT2 (red) and actin labeled with AF647-phalloidin (green) in fixed NRK52E cells. In (B) EGFP-SEPT2 was labeled with CF680 via anti-GFP nanobodies.
Figure 6.6: **Two-color SMLM of septin filaments in NRK52E cells.** (A) Reconstructed superresolution image of EGFP-SEPT2 labeled with anti-GFP nanobodies (green) and SEPT7 labeled with primary and secondary antibodies (red). (B) Representative filament (top) and its intensity profile in two colors (bottom). (C) Spatial cross-correlation analysis of SEPT2 and SEPT7 (SEPT2×SEPT7) averaged for 52 filaments from three different cells.
antibodies. The resulting two-color SMLM images showed, that both septins were incorporated into the same filaments (Figure 6.6 A). Moreover, the localizations in two colors were alternating in a seemingly regular fashion along filaments, confirming the end-to-end assembly of rod-like septin complexes (Figure 6.6 B).

Spatial cross-correlation analysis between SEPT2 and SEPT7 localizations of the two-color superresolution images, averaged over 50 filaments from 3 cells, showed approximately 8 nm, 32 nm and 80 nm periodicities. The detected shortest 8 nm correlation distance between SEPT2 and SEPT7, is in a good agreement with the structure of both hexamers and octamers and was also observed using overexpression of SEPT2-EGFP and SEPT7-mCherry (see Section 4.1.2).

6.2 Conclusions

The structural analysis of filamentous septin higher-order structures was performed by superresolution microscopy in genome edited NRK52E cells where endogenous SEPT2 was tagged with EGFP. The staining method based on anti-GFP nanobodies provided highly efficient and proximal labeling. In superresolution images, the central SEPT2-dimer within the complex exhibited chains of localizations along filaments with regular spacing, suggesting that in vivo complexes assemble end-to-end into strands in accordance with in vitro studies. Further analysis of the periodicity of SEPT2 occurrence along filaments showed that single-molecule localization-spots along individual filaments were highly ordered with varying periodicity between different filaments. The observed average period of 50 nm was substantially higher than the ~27 to 35 nm expected from the model for hexameric and octameric septin complexes. Considering that as many as 30 septin homologs could be expressed in mammalian cells, larger and more complicated complex assemblies may be possible. The results show clearly that the issue of septin complex size and stoichiometry is more complicated than expected and requires further study.
Chapter 7

Subunit exchange of membrane-associated septin assemblies

According to the current model, septins in the cytosol polymerize into complexes in a hexameric and/or octameric organization. The hexamer has the following order: SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7; in the octameric complex the hexamer is flanked by SEPT9 on both ends. These complexes arrive at the plasma membrane from the cytoplasm where they then form higher-order structures by annealing laterally or longitudinally. Higher-order filamentous septin structures are associated with F-actin or microtubules in interphase mammalian cells, but appear to be less dynamic than conventional cytoskeletal proteins. If and how individual subunits exchange in already formed filaments remains unclear. The canonical model of hexameric and octameric septin complexes suggests that septins exchange exclusively as entire complexes, however, work of Hagiwara and coworkers showed that septins exchange within filament as monomers [35]. In contrast, in another study it was proposed that the septin exchange unit is a pair of complexes [16]. It is not yet understood how septins from the cytosol are incorporated into membrane-associated filamentous assemblies in live cells. Evidently, the role of individual septins or septin complexes in septin filament dynamics in vivo requires further study.
This chapter will cover the application of single-molecule microscopy of genome-edited cell lines to elucidate the stoichiometry and dynamics of fluorescently labeled SEPT2 proteins. Genome-edited cell lines provide an excellent tool for quantitative fluorescence microscopy by a method termed stepwise photobleaching. The method relies on the irreversible and stochastic loss of fluorescence due to changes in the fluorophores structure following a light-induced chemical reaction. For an individual fluorophore, photobleaching is observed as a stepwise drop in fluorescence intensity and for multiprotein complexes the number of observed photobleaching steps can give access to the protein stoichiometry. This powerful method has been used, for instance, to determine the stoichiometry of transmembrane proteins [108] or complexes of other types including β-Amyloid [20].

The stoichiometry of SEPT2-EGFP molecules in septin complexes can be resolved by counting the number of photobleaching steps of EGFP within a single complex. Since each SEPT2 within a complex is tagged with EGFP, the number of photobleaching steps provides access to the number of SEPT2 molecules within the complex. Moreover, the number of SEPT2 subunits within a diffraction-limited fluorescent spot can be deduced from the fluorescence intensity by comparison to the brightness of a single EGFP molecule. Here, a series of stepwise bleaching experiments of SEPT2-EGFP were performed in live cells and in vitro by pull-down of different septin complex subunits from whole-cell lysate.

Besides the stoichiometric information contained in fluorescence intensities, additionally the positional information from the localization of the single EGFP-SEPT2 molecules was used to study the localization and diffusion dynamics of individual septin complexes. The diffusion dynamics of SEPT2-EGFP molecules newly arriving at the plasma membrane was analyzed by uPAINT (described in Section 3.1.2). This provided new insight into process of subunit exchange and lengthening of already formed septin assemblies in their native and live-cell environment.
7.1 Results and Discussion

7.1.1 SEPT2 exchange unit in live cells

Stepwise bleaching experiments were performed on genome-edited epitheloid NRK52E cells on the custom-built single-molecule sensitive setup. Imaging was performed using TIRF where the illumination is restricted to only \(\sim 200 \text{ nm} \) above the coverslip surface. Consequently, the EGFP-SEPT2 molecules were excited almost exclusively at the ventral cellular plasma membrane thereby minimizing photobleaching of EGFP-SEPT2 pool in the cytoplasm. TIRF illumination also facilitated the observation of septins on the membrane by suppressing out-of-focus fluorescence produced by the cytosolic EGFP-SEPT2 as well as cellular autofluorescence. Under moderately low blue-laser illumination-intensity, septin filaments at the plasma membrane were bleached over time to allow for the observation of separated diffraction-limited fluorescent spots appearing in these filaments by exchange with the cytoplasmic pool. The fluorescence of these EGFP tagged molecules newly arriving at the membrane from the cytosol was then recorded (see Section 8.8 for detailed methods).

Due to similar rates of photobleaching and arrival of new EGFP molecules, the density of observed EGFP molecules per single frame was consistently low enough to observe single fluorescent spots throughout the acquisition. A single experiment of only a few minutes of acquisition provided hundreds of fluorescent spots with heterogenous intensity from a small subregion of the cell.

By localizing the centroid of the fluorescent spots it was possible to reconstruct a super-resolution image, which confirmed that the majority of spots indeed localized to filamentous septin structures (Figure 7.6).

The large number of events offered the possibility for quantitative statistics, but also required a robust, automated method for analyzing the fluorescence trajectories. This would also reduce any user-induced bias in the decision if a particular spot is fit for the analysis, for instance by favoring brighter events (which are more likely to consist of multiple EGFPs), or bias in the interpretation of the fluorescence trajectory in terms of the number of observed photobleaching steps. Towards this goal, a software-based automated detection of fluorescent spots from
PART III. SINGLE-MOLECULE MICROSCOPY OF SEPTINS

camera frames and determination of photobleaching steps from the resulting intensity traces was developed.

First, all spots were detected using single-molecule localization analysis described previously in Section 8.1. A very low photon-count cutoff was chosen for the localization in order to detect all possible arriving events, which resulted in millions of localizations per single 2-3 min movie. To discriminate localizations arising from background noise or autofluorescent contaminants on the plasma membrane from those corresponding to EGFP-SEPT2, events with a distance smaller than 1.5 camera pixels (∼100 nm) between consecutive frames and a maximum time-gap of one frame were clustered. Only clusters with a length of over 40 frames were considered for analysis. The parameters for clustering were chosen empirically based on the fact that membrane-associated EGFP-SEPT2 molecules in some cases were moderately mobile and exhibited minor blinking behavior. This resulted in 200-300 clusters suitable for analysis of photobleaching steps per movie. The fluorescence intensity-traces over time for selected clusters were computed from the pixel-intensities of the raw image sequences.

The integrated fluorescence of the spots was determined from a 7 × 7 pixel region centered on the coordinates of spots determined by the localization software. In this way, the mobile behavior of fluorescent spots in the membrane of live cells was taken into account. The noisy intensity traces were first smoothened using the edge-preserving Chung-Kennedy filter [13] and then processed with the Canny one-dimensional edge detector [11]. In this way, the segments of constant intensity, followed by sharp changes were identified and the number of intensity-steps per fluorescence trajectory could be extracted. The principle of the experiment is illustrated in Figure 7.1.

Experiments in NRK52E cells showed that 43% of the fluorescent spots on the membrane bleached in a single step while 57% bleached in two steps. This initial observation is already a very strong indication that the fluorescent spots consist of at least two SEPT2 subunits arriving to the membrane in pairs (Figure 7.2). The large proportion of single steps arises from the fact that some of the EGFP molecules may photobleach in the cytosol before arriving to the membrane despite the small penetration depth of the TIRF illumination. Moreover, a substantial proportion of EGFP molecules are non-fluorescent from the beginning due to
Figure 7.1: **Stepwise photobleaching of EGFP-SEPT2 in live NRK52E cells.**

(A) Schematic of the experimental configuration showing a pre-bleached region of the basal membrane of a live cell illuminated in TIRF mode. The fluorescence of EGFP molecules newly arriving from the cytosol is recorded. (B) Coordinates of fluorescent spots identified by single-molecule localization fulfilling the clustering criteria. (C) A sample frame of a recorded image sequence where a blue circle and green squares outline spots that exhibited one and two-step photobleaching, respectively. (D) Example intensity-traces of the spots marked in (C). Raw data in green for two-step traces and in blue for the spot exhibiting a single step. The red lines are the plateaus of intensity identified by the step-counting algorithm. (E) Fluorescence intensity images of the sequence corresponding to spot number 2 in (C). Pixels are 60 nm.
misfolding or incomplete maturation in the cell environment. As much as 20-53% of non-fluorescent EGFP has been reported in different studies [32, 66, 108].

Under the assumption of a constant stoichiometry of EGFP-SEPT2 molecules in the fluorescent spots at the membrane, the numbers of detected photobleaching steps should display a binomial distribution. Therefore, the acquired data was compared to the binomial distributions for a number of feasible stoichiometries (monomeric, dimeric, tetrameric). With the probability of 0.75 of the EGFP-tag being properly folded, fluorescent and therefore producing a step, the data could be adequately reproduced by the binomial distribution for a dimer (Figure 7.2 B). Hence, the model with two SEPT2 subunits in the septin exchange unit reflects the experimental data the best. To additionally support these findings, the number of EGFP-SEPT2 molecules in a fluorescent spot was deduced from the distribution of fluorescence intensities of the spots.

The fluorescence intensity of individual spots depends on the illumination profile over the field of view, as well as on intensity variations due to dipole orientation of the fluorescent molecule, which can be as high as 35% as reported for fluorescent proteins observed in the membrane of live mammalian cells [32]. For very close fluorophores another problem may arise from self-quenching due to homo-FRET. The comparison between the overall drop in fluorescence intensity between double-step and single-steps events showed a roughly two-fold higher (1.9 ± 0.2) drop in fluorescence intensity for the two-step bleaching events Figure 7.2 C. This was in good agreement with the results obtained from the step-analysis. Therefore, it was established that the exchange unit of septin filaments in their native environment includes a SEPT2-SEPT2 dimer rather than SEPT2 monomer or a pair of dimers.

7.1.2 Stoichiometry of SEPT2 in physiological septin complexes

in vitro

While the live cell experiments provided strong evidence that the exchange unit of membranous septin assemblies contains a SEPT2 dimer, they could not determine whether the SEPT2 dimers alone represent the unit of exchange, or if they exist in octameric complexes. To complement the results from live-cell imaging, the
Figure 7.2: **Stepwise bleaching statistics of EGFP-SEPT2 in live cells.** (A) Histogram of observed steps for two independent experiments (white bars) compared to the binomial distribution for 1, 2 or 4 GFP-tagged subunits. (C) Intensity based analysis of SEPT2 stoichiometry. Histogram shows distribution of the overall drop in fluorescence intensity for single and double-step bleaching events.

Stoichiometry of SEPT2 in physiological septin complexes was further studied *in vitro*. A single-molecule pull-down assay [48] was employed in combination with stepwise photobleaching to determine the presence and number of EGFP-SEPT2 molecules in SEPT9 and EGFP-positive complexes. In these experiments, antibodies either against EGFP at the central SEPT2 or against SEPT9 at the ends of the octameric complex were immobilized on microscope coverslips and cell extracts from the genome-edited cell line were applied. Captured EGFP was imaged using single-molecule TIRF microscopy and the discrete photobleaching steps of fluorescent spots were analyzed as described in Section 8.8.

Selective immobilization of septin complexes from cell lysates was achieved via the biotin-streptavidin tag in order to minimize the disturbance to protein structure. Commercially available microscope glass coverslips were used, which were passivated with poly-ethyleneglycol (PEG) to reduce non-specific adsorption and coated with streptavidin at low density. The surfaces were activated via biotinylated anti-GFP antibodies or indirectly via the biotinylated secondary antibodies towards primary anti-septin antibodies.
For successful single-molecule imaging, the proteins were immobilized at very low densities to ensure that only one molecule is present within a diffraction-limited area. This was achieved by 20 min incubation of the activated surface with low picomolar concentrations of the fluorescent probe (cell lysate or purified EGFP molecules). The surface-coverage of antibodies on the surface was much higher than the density of immobilized proteins (this was determined by saturation of the surface), so that protein capture was effectively not limited by the availability of binding sites. This ensured a sparse distribution of fluorescent spots on the surface and a minimal probability of having two targets bound to the bivalent capture antibodies.

To test the quality of the coverslips for single-molecule experiments and to estimate the number of background counts, either purified EGFP at low picomolar concentration in dilution buffer (+GFP) or dilution buffer only (-GFP) was applied to the activated surface for 20 min (Figure 7.3 A,B). After washing, the samples were imaged using identical conditions and analyzed in terms of the fluorescence-intensity profiles of detected spots. Though the control experiment without GFP showed fluorescent spots, it was on the order of 10% of the number spots detected for the GFP-positive control. A comparably low number of fluorescent spots were observed for the control lysate from wild type cells in comparison to the lysate from genome edited NRK52E cells (Figure 7.3 C). From this it could be concluded that cellular autofluorescence did not pose a significant problem and it was proceeded with the actual pull-down experiments and stepwise bleaching analysis.

Figure 7.4 shows a single-molecule pull-down and stepwise bleaching experiment performed using cell lysate from genome edited NRK52E cells with anti-GFP antibody activated surface.

The distribution of counted steps for the pull-down of EGFP-SEPT2 from whole-cell lysate showed 46% of the spots photobleaching in one step, 45% in two, 6% and 2% in three and four steps, respectively (Figure 7.5 C). This distribution follows the binomial distribution for a EGFP dimer better than the model of a monomer or tetramer. The large number of spots with two bleaching steps is consistent with the results of stepwise photobleaching experiment in live cells.

Finally, the stoichiometry of EGFP-SEPT2 in SEPT9 positive complexes was assessed. For this, septin complexes from the cell lysate were immobilized in-
Figure 7.3: **Quality control of activated surfaces.** The first frame of an image sequence of a coverslip without (A) and with immobilization of purified GFP (B). (C) Histogram of the number of fluorescent spots for: dilution buffer in absence of purified GFP (-GFP), dilution buffer with GFP (+GFP), lysates from wild-type NRK52E cells (wt-Lysate), lysate from genome-edited NRK52E (Lysate), and for coverslips incubated with purified GFP without immobilization of anti-GFP antibodies (-αGFP ab).

7.2 **The dynamics of septin complexes at the plasma membrane**

Recent experiments conducted in *A. Gossypii* suggest that septins arrive as assembled complexes from the cytosol at the plasma membrane where they diffuse freely until they collide with other complexes or filaments to anneal and form larger assemblies [8]. Little is known about the dynamics of septin complexes...
Figure 7.4: **Stepwise photobleaching of EGFP-SEPT2 from cell lysate.** EGFP-SEPT2 molecules in the cell lysate of genome edited NRK52E cells captured via anti-GFP antibodies. (A) The first frame (t = 0 s) and the last frame (t = 120 s) of the image sequence. Blue circles and green squares denote examples of fluorescent spots that exhibited one- and two-step photobleaching, respectively. (B) Corresponding intensity traces where the red lines represent intensity-plateaus detected by the step-counting algorithm. (C) Fluorescence intensity images of the sequence corresponding to the green square no. 3 in (A) displaying two-step photobleaching.
Figure 7.5: **EGFP-SEPT2 stoichiometry from stepwise bleaching on whole-cell lysates.** Shown are the step histograms for the pull-down experiments on genome-edited NRK52E cells using immobilized anti-GFP antibody (top row) and anti-SEPT9 antibody (bottom row). (Left column) Schematic of the specific pull-down experiment. (Middle column) Distribution of observed steps for a number of independent experiments. (Right column) Distribution with standard deviation of observed steps averaged over all experiments compared a binomial distribution for a EGFP monomer, dimer and tetramer for a probability $p = 0.75$ of producing a step.
and higher order septin filaments in the plasma membrane of mammalian cells.

In the following, the dynamics of EGFP-SEPT2 complexes newly arriving at the membrane of live cells was investigated by live-cell single molecule imaging and provided access to their motility as well as the rules governing their exchange within larger septin assemblies.

While it was shown in the previous sections that the exchange unit for preexisting septin filaments are complexes, in the case of mammalian cells, it still remains unclear how they incorporate at sites of the preassembled filaments. The assembly dynamics were investigated using a variation of the single-molecule technique uPAINT.

Here, the fluorescent spots from continuously arriving and bleaching EGFP-SEPT2 molecules was used to reconstruct a superresolution image by single-molecule localization. Rendering of all registered events provided images of membranous structures, which were comparable to the super-resolved short curved septin filaments on the membrane of fixed cells as shown in Chapter 6. When the localizations were represented using time-coded colors, the images displayed no preferential addition of subunits to the filament ends. This observation is in contrast to the observations made in yeast [8] where septin filaments were only seen to be elongated at their ends.

The single-molecule position information also allowed study of septin dynamics on the membrane by tracking the newly-arrived EGFP-SEPT2 molecules over time. Individual events localized with a precision below 30 nm a maximal step-size of of 300 nm between consecutive frames, and interrupted by not more than two frames, were grouped into diffusion tracks. These tracks are visualized in Figure 7.6 B.

Many septin molecules which were analyzed showed a similar dynamic pattern: faster diffusion within a short range of approximately 250 nm in the beginning of a track, followed by slower, confined diffusion. The mean square displacement (MSD), a parameter that characterizes the typical area the molecule covers during its diffusional path, was plotted over time. It shows an initial steep linear increase during the first 200 ms, followed by a second, lower linear increase on the longer time-scales (Figure 7.6 C). It shows an initial phase of rapid diffusion followed by a second phase with a dramatic decrease in the speed of diffusion. This
ensemble observation is in good agreement, with what is observed for the behavior on the single-molecule level. From the MSD plot over time two separate diffusion coefficients were determined: \( \sim 5.0 \times 10^{-2} \, \mu m^2/s \) for the fast diffusion, which is in the range observed for the free membrane molecules, \( \sim 4.8 \times 10^{-3} \, \mu m^2/s \) and for the slow diffusion, which describes the diffusion of larger septin assemblies.

### 7.3 Conclusions

It was demonstrated here in stepwise bleaching experiments, performed in live genome-edited cells, that the exchange unit of septin filaments in their native environment includes a SEPT2-SEPT2 dimer as opposed to individual SEPT2 monomers. This also rules out an exchange unit consisting of a pair of complex-dimers as it has previously been suggested on the basis of the more indirect method of fluorescence polarization anisotropy [16], since such a structure would contain four SEPT2 molecules. The \textit{in vitro} pull-down experiments using lysate from genome-edited cells targeted towards the complex end-capping SEPT9 suggests that, indeed, the observed SEPT2 dimers reside in assembled octameric complexes. Using the time-resolved sub-diffraction position information of EGFP-SEPT2 in live cells additionally established how these complexes incorporate into assembled filaments on the membrane after arrival from the cytosolic pool. The experiments showed that complexes arriving at the membrane diffuse freely on short length-scales where their mean free path of a few hundred nanometers is consistent with the filament density we observed by live-cell superresolution imaging. It was furthermore found that the complexes can incorporate over the entire length of preexisting filamentous septins. This is remarkably different to what has been reported for yeast [8], where septin filaments were only seen to be elongated from their ends.
Figure 7.6: The dynamics of septin filament formation in live cells. (A) Localizations of individual events of EGFP-SEPT2 molecules were used to reconstruct a superresolution image of septin organization. (B) The corresponding trajectories of EGFP-SEPT2. The colors are time-coded. (C) A close-up of three septin filaments. On the left the superresolution image, in the middle the same image with the time-coded color assignment, on the right the corresponding diffusion tracks with the same colors. (D) Mean square displacement plot over time for 3635 single-molecule trajectories. Only tracks with a minimum length of 5 frames were considered and the average track-length was 11 frames. The diffusion coefficient was determined from the slope of the linear regression using $\langle \text{MSD} \rangle = 4D\Delta t$. 

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Part IV

Materials and Methods
8.1 Microscopy setups

Immunofluorescence microscopy was performed on a spinning-disk confocal setup (Roper Scientific) on an inverted Olympus IX71 while SMLM was performed on custom microscope setup shown in Figure 8.1.

Figure 8.1: Custom SMLM setup. (A) Simplified overview of the setup. D1-D4: dichroic mirrors, AOTF: acousto-optical tunable filter, TL1/TL2: telescope lenses (exchangeable), TFL: TIRF lens on linear translation stage, λ/4: lambda quarter plate, BFP: objective back focal plane, PL1/PL2: propagation lenses, F: emission filters, TL: tube lens, EMCCD: electron-multiplying charge-coupled device. Not included in the scheme are: excitation filters, laser beam-expanders, the focus-lock system and the z-piezo at the objective. (B) Simplified overview of changes to the emission path for bi-plane and/or dual color imaging. F: emission filter, LP-DM: long-pass dichroic beamsplitter, BS: 50:50 non-polarizing beamsplitter, M: mirrors, L1: tube lenses, L2: tube lens on linear translation stage, FP1/FP2: focal planes, EMCCD: electron multiplying charge-coupled device. For two-color 2D imaging the focal planes FP1 and FP2 are equal. For single-color 3D imaging the dichroic beamsplitter LP-DM was replaced by a non-polarizing beamsplitter BS.

A 405 nm (50 mW, Laserglow Technologies), 473 nm (100 mW, Laserglow Technologies), a 556 nm (200 mW, Laserglow Technologies) a 643 nm laser (150 mW, Toptica Photonics), and an auxiliary 643 nm laser (200 mW, Laserglow Technologies) were expanded, cleaned up with 30 pinhole, and focused onto the back-focal plane of an Olympus 60x, Plan Apo, NA 1.49, TIRF-objective. The maximum power at the microscope objective was 2.5 kW/cm² for 643 nm
and up to 5.6 kW/cm² with the auxiliary red laser, 2.5 kW/cm² for 556 nm, 0.2 kW/cm² for 473 nm and 0.05 kW/cm² for 405 nm. A quad-edge dichroic beamsplitter (405/488/532/635 nm, Semrock) was used to separate fluorescence emission from excitation light. Emission light was filtered by a quad-band bandpass filter (446/523/600/677 nm, Semrock). Bandpass emission filters were used (blue excitation: ET525/50 M, Chroma, green excitation: 607/70, Semrock, red excitation: ET700/75, Chroma).

For two-color imaging by spectral demixing two bandpass emission filters (ET700/75 nm, Chroma) and a longpass dichroic beamsplitter (690 nm, AHF Analysetechnik) were employed. The emission light was focused by two separate 500 mm tube lenses onto a back-illuminated EM-CCD camera chip (Evolve, Photometrics) which was liquid cooled to -80 °C. Focusing was done by moving the objective with a piezo objective positioner (MIPOS100, Piezo Systems Jena). A focus lock was implemented by an electronic feedback loop. It was based on total internal reflection of a red laser at the cover slip and its detection on a quadrant photodiode. The z-stability was better than ±10 nm over several hours. The sample stage was xy-translatable and could be heated to 35 °C for live-cell applications. Instrument control was done with software written in LabVIEW and image acquisition was performed with MicroManager. Single-molecule localization was done using a maximum-likelihood estimator fitting routine using Gaussian point spread functions [99] implemented on graphics processing unit (GPU) for fast fitting. An image-correlation based drift-correction was employed.

8.2 dSTORM imaging procedure

For single-color imaging using Alexa Fluor 647, the switching buffer consisted of 130 mM 2-mercaptoethanol/0.2 M Tris, pH 8.0 with the GLOX oxygen-scavenging system (5% (w/v) glucose, 0.25 mg/ml glucose-oxidase and 20 μg/ml catalase). In the case of two-color imaging with Alexa Fluor 647 and CF680 100 mM mercaptoethanolamine/0.2 M Tris, pH 8.0 and GLOX was used as blinking buffer. The duty-cycle, especially of CF680, was substantially better in this buffer, however, at the expense of 50% fewer photons per switching cycle for both dyes. The intensity of either 405 nm or 473 nm activation laser was automatically adjusted by
software to keep the average number of localizations per frame constant. Imaging
was generally performed in objective-type total internal reflection fluorescence
(TIRF) mode using a penetration depth depending on the sample. A minimum
of 20,000 frames with exposure times between 25-50 ms were recorded. The
imaging laser intensity of the 643 nm laser line used was typically \( \sim 2 \text{ kW/cm}^2 \).

### 8.3 Two-color spectral-demixing and 3D image analysis

All image analysis was performed with in-house MATLAB software. The first
few tens of frames of the dSTORM acquisition were used for a coarse estimate
of the translational shift between the left and right side of the camera chip. This
was done using the phase-difference of the Fast Fourier Transform which was
calculated from the pixel-reconstructed image (\( \sim 100 \text{ nm pixel size} \)). The coarsely
aligned localizations were paired where the localizations from the two channels
had to be within a maximum search-radius of 8 camera pixels (\( \sim 800 \text{ nm} \)). The
paired localizations were used to calculate an affine transformation matrix using
the MATLAB built-in routine `cp2tform`. The obtained transformation matrix was
then applied to map all localizations from one side of the chip onto the other side.
Now all localizations were paired using a more stringent cutoff of 3 pixels for
the search-radius. The normalized intensity ratios were then calculated for all
localization pairs for the color-assignment using \( r = (I_s - I_l) / (I_s + I_l) \), where \( I_s \)
and \( I_l \) are the fluorescence intensities, determined by maximum-likelihood fitting,
the short and long wavelength channels. For two-color 3D imaging based on bi-
plane, relative z-positions were calculated according to \( \Delta z = \sigma_l^2 - \sigma_s^2 / m \), where
\( \sigma_l^2 \) and \( \sigma_s^2 \) are the widths of the fitted PSFs of the localization pair for the long
and short wavelength channel, and \( m \) the slope of the calibration curve determined
from a z-stack of fluorescent nanobeads.
8.4 Correlation analysis of septin filaments

Individual septin filaments were manually cropped from the single or dual color SMLM images. The localizations were grouped according to \( dX = 1, dT = 10 \). Each filament was straightened by fitting the localizations of the combined channels with 5\(^{th}\) order polynomial function. The y-position value for the straightened filament was obtained from the shortest distance of the localization to the polynomial function, the x-position value from the running contour-length along the filament. The localizations were converted from coordinates to pixel intensities with 1 nm pixel-size. The images were low-pass filtered with a Gaussian filter with 1 pixel standard deviation and a 3\(\times\)3 pixel filter kernel. The \( m \times n \) pixel images were converted to an \( m \times 1 \) vector by summing the pixels perpendicular to the filament major axis. The normalized autocorrelation or cross-correlation on the vectors was computed using the MATLAB built-in routine `autocorr` or `crosscorr`.

8.5 Radial distribution analysis of septins

The superresolution images were converted from localization coordinates to pixel intensities with 5 nm pixel size. The radial distribution function was calculated using an open source available ImageJ Macro (http://rsb.info.nih.gov/ij/plugins/radial-profile.html).

8.6 Labeling of nanobodies and antibodies

Anti-GFP- and anti-RFP nanobodies (Chromotek) were labeled with Alexa Fluor 647 (Life Sciences) and CF680 (Biotium) by standard N-hydroxysuccimidyl ester chemistry according to the manufacturer’s protocol using 5-fold molar excess of dye. The anti-mouse antibodies were labeled accordingly with CF680 using an 8-fold molar excess of dye. Nanobodies and antibodies were purified using three subsequent passages through 7 kDa MWCO desalting columns (Thermo Fisher). The dye to nanobody labeling-ratio was 1.0-1.5:1 and the dye to antibody labeling-ratio was 2.5:1 as verified by UV/Vis absorption spectrometry according to the
manufacturer’s protocol. Antibodies were labeled using the same procure but using a 15 fold molar excess of dye. The resulting dye to antibody labeling-ratio was ∼3-5.

8.7 Cell culture, transfection, and immunostaining

NRK, U2OS, Ptk2 and CV1 cells were grown in low glucose DMEM without phenol red supplemented with 10% fetal bovine serum, penicillin, streptomycin and GlutaMAX (all Life Technologies) at 37°C in a CO2-controlled humidified incubator. Cells were transferred to round 18 mm diameter #1.0 glass coverslips (Menzel) and transiently transfected with Lipofectamine 2000 (Life Technologies). The cells were fixed 18-48 h after transfection. For imaging of microtubules cells were extracted for 1 min in 0.5% (v/v) Triton-X100 (Sigma) in BRB80 (80 mM PIPES, 1 mM MgCl2 and 1 mM EGTA (all Sigma), pH 6.8) and fixed for 5 min with Methanol (VWR) at -20°C. For Cav1 and septin imaging the cells were fixed for 10 min with 4% Paraformaldehyde/2% Sucrose (Sigma) in BRB80. The coverslips were washed in PBS or BRB80 as indicated, incubated for 15 min in 50 mM NH4Cl, blocked with Image-IT (Life Technologies) for 30 min, blocked with 4% horse serum/1% BSA for 1 h and incubated with ∼5-20 nM nanobodies for 1 h. For antibody staining, blocked cells were incubated for 1 h with monoclonal mouse-anti-β-tubulin antibody (Sigma T5293), washed with BRB80 and incubated with either Alexa Fluor 647 goat-anti-mouse antibody (Invitrogen A21236) or custom-labeled CF680 goat-anti-mouse (M30100 Invitrogen) for 1 h.

Generally, the sample preparation for imaging via nanobodies consists of several simple steps and can be performed in 2.5 h. This makes it very easy to perform SMLM measurements on the same day to avoid deterioration of the sample. We noted that nanobody-staining performed equally well after fixation with most common reagents such as paraformaldehyde, glutaraldehyde, or methanol, when the measures to reduce fixative specific background are taken. This is important because some structures such as actin or microtubules require specific staining conditions and not all antibodies are compatible with glutaraldehyde or methanol fixation. The overall steps of the labeling protocol are visualized in a Diagram (Figure 3.3)
PART IV. MATERIALS AND METHODS

8.8 Live cell microscopy for stepwise bleaching

Imaging was performed using the setup described in Section 8.1 equipped with a high numerical aperture objective (Olympus, 100x, Apo, NA 1.65) which provides an approximately 50% higher collection efficiency compared to the NA 1.49 objective. The cells were cultured on quartz coverslips and imaging was performed at 35°C in live-cell imaging buffer (145 mM NaCl, 5 mM KCl, 10 mM Glucose, 10 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂, 0.2% (w/v) BSA, 10 mM ascorbate). A moderately low illumination intensity was used that provided a good single-to-noise ratio, bleached arriving EGFP-SEPT2 molecules after several seconds, albeit sparing EGFP molecules in the cytosol. An exposure time of 50 ms was used and image sequences of 1000-3000 frames were recorded for subsequent analysis.

8.9 Cell lysis

NRK52E-SEPT2-EGFP cells were grown in 10 cm dish to 70-90% confluence. All further steps were performed at 4°C. Cells were rinsed with PBS and scraped off the plate in 1 ml PBS and spun down at 500 g for 3 min, was washed twice with PBS and resuspended in 200 μl lysis buffer (10 mM Tris pH7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40 with protease inhibitors and 1 mM PMSF) and passed through a 22 gauge needle and incubated for 30 min, and mixed thoroughly every 10 min. The lysate was centrifuged at 20,000 g for 10 min. Lysate supernatants were transferred to a pre-cooled tube or frozen for long-term storage at -80°C.

8.10 Single-molecule pull-down of septin complexes from cell lysates

The streptavidin coated glass coverslips were purchased from MicroSurfaces, Inc and kept in the vacuumed bag at -80°C. Once opened, the coverslips were used within several hours or kept overnight in a clean desiccator under vacuum. The 18 mm squared coverslips were glued to the media chambers of 8-well Glass
Slide Lab-Tek chambers with Evo-Stik epoxy (Bostik), which was tested to be non-toxic for live cells. In this way, one coverslip was used for four independent experiments. All incubation steps were performed by pipetting 50 μl to the well and all wash-steps were performed by pipetting three times 400 μl to the well. All dilutions and washing steps were performed in Tris/DTT buffer (200 mM TrisHCl pH 7.5, 1 mM MgCl2, 1 mM DTT). The antibodies and purified EGFP were diluted to 100 ng/ml directly before application to the surfaces. The coverslips were incubated with biotinylated monoclonal mouse anti-GFP (A10259, Life Technologies) or in-house biotinylated secondary anti-rabbit antibodies (R2004, Sigma) for 60 min. In the case of secondary antibodies, after a round of washing the chambers were incubated with polyclonal rabbit primary anti-SEPT9 antibodies (R39208, Sigma) for 60 min, followed by several rounds of washing. The cell lysates were diluted 1:1000 directly before applying to the activated surfaces. The final step, pull-down of purified proteins or proteins from the cell-lysate, was performed on the microscope stage. The binding of EGFP was monitored under low blue laser illumination and roughly after 20 min stopped by extensive washing.

8.11 Fluorescent spot detection and analysis of fluorescent trajectories

The detection of fluorescent spots was performed using the localization analysis described in Section 8.1. The detected localizations with a distance smaller than 1.5 camera pixels in consecutive frames (interrupted by not more than one frame) were grouped in a single cluster. Only clusters containing more than 40 frames were considered for further analysis. Obtained coordinates of localizations were used to calculate the integrated intensity from the camera images using \(7 \times 7\) pixels for the 100x NA 1.65 objective in live-cell imaging, or \(5 \times 5\) pixels for the 60x NA 1.49 objective in \textit{in vitro} experiments. The resulting intensity traces of fluorescent spots over time were first smoothed with a Chung-Kennedy filter [13] that has previously been shown to be the most appropriate for detecting step-wise changes in single-molecule fluorescence [58]. The filter avoids the edge blurring by calculating the mean and standard deviation across backward and forward
non-overlapping windows of data. The output is the mean of the window with the lowest standard deviation. The result of the calculation for every data point in a trace is a running average that is still capable of preserving sharp changes in signal amplitude (Figure 8.2 A). The input parameter, the size of the running window, of 4 data points was used throughout the analysis.

Figure 8.2: **Signal processing for step-size analysis.** (A) A sample intensity trace smoothened by the edge-preserving Chung-Kennedy filter. (B) Canny edge-detection with a coarse (left) and fine (right) threshold which was set according to the amount of noise in the image. C. The segments of constant intensity were filtered depending on the difference in height between adjacent segments.

To detect the segments of constant intensity the one-dimensional Canny edge-detector was used. The MATLAB code was adapted from Ref. [44]. It involves further noise suppression by convolving the traces and an edge is characterized as a point where the first order derivative of a Gaussian kernel is large and the second order derivative minimal. The scale parameter of the Gaussian kernel selects the degree of noise suppression and must be tuned to appropriate values based on the noise of the data. The fine thresholding was found to be the most appropriate for the experimental intensity traces (Figure 8.2 B). The identified segments of constant intensity were additionally filtered according to the step height to reduce the number of false-positive steps. Adjacent segments with a height difference of less than 10% of the difference between the highest and lowest plateaus were averaged. This was performed iteratively (Figure 8.2 C). These parameters for the procedure were found empirically.
**8.12 Binomial model for stepwise bleaching**

The observed distribution of photobleaching steps was compared to a binomial distribution. When $M$ is the total number of fluorescent subunits in a single fluorescent spot and $P$ is the probability of a fluorophore to be functioning, the probability $q_n$ of observing $n$ steps in the time-trace is:

$$q_n = \frac{M!}{n!(M-n)!} P^2 (1-P)^{M-n} \quad (8.1)$$

For all calculations $P = 0.75$ was used and the resulting binomial probability mass function was multiplied by the total number of detected spots $N$ to get a semi-scaled simulated distribution of step-sizes. Note, that this approach is not entirely correct since $N$ does not represent the number of observations as a 0-step cannot be observed.

**8.13 Single Particle Tracking**

For SPT measurements, molecules selected for particle tracking were required to have lateral localization precision better than 15 nm for AF647 and 40 nm for EGFP in each frame. Localizations with distance smaller than ~500 nm in consecutive frames were grouped into a track and only tracks with a minimum length of 20 consecutive frames were considered for analysis. For each track a diffusion coefficient $D$ was calculated from the mean square displacement (MSD) using the following relationship:

$$\text{MSD}(t) = \left< r^2 \right> = 4 \cdot D \cdot t, \quad (8.2)$$

where $r^2$ is $(x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2$ in the $i$-th frame, $D$ is the diffusion coefficient and $t$ is the elapsed time between two successive frames. The diffusion coefficient $D$ was calculated from the slope of a linear fit through the points of the MSD plotted versus $t$. In case of the SPT measurements of EGFP-SEPT2 the values of $D_{\text{fast}}$ and $D_{\text{slow}}$ were calculated based on two apparent linear segments of the MSD plot.
PART IV. MATERIALS AND METHODS
Summary and Outlook

In this work, a labeling strategy was developed to addresses several fundamental issues in superresolution fluorescence microscopy. Accurate delivery of suitable fluorophores into close proximity of the target structure without introducing background is a common difficulty. Sample labeling can be hampered by the size, the lack of specificity or the availability of the labeling agent. A labeling method was established that relies on the expression of recombinant constructs tagged with GFP and RFP-derivatives which serve as epitopes for nanobodies coupled to bright fluorescent probes suitable for SMLM. The nanobodies provide precise consistent labeling, high accessibility in dense cellular structures, together with a fast and simple procedure for preparation of the sample. The quality and versatility of the method was demonstrated by performing superresolution imaging of various nanoscopic structures in cells, including microtubules, caveolae and nuclear pore proteins. Applications were extended to single-particle tracking of membrane molecules in live neurons. Our technique would be especially powerful in combination with the many available GFP-knock-in mice. It also brings a possibility to efficiently label targets in the optically thick tissue slices, hence the super-resolution imaging will be a complimentary method to EM to investigate various cellular processes in vivo.

Towards the investigation of multiprotein complexes, the developed labeling strategy was further extended to provide a simple and highly generic technique for two-color superresolution imaging. With the use of only two nanobodies against GFP and RFP, the method allows for the investigation of the relative organization of virtually any combination of the widely available functional fluorescent protein fusion constructs. These benefits were combined with the use of a powerful imaging configuration for two-color superresolution together with an advanced
dye pair. The employed spectral-demixing method helps overcome a number of widespread difficulties related to two-color imaging, such as color-crosstalk, chromatic aberration, color registration and buffer compatibility of the dyes. Moreover, its implementation requires only minimal technical demands and provides a compound resolution close to the limit commonly reached only in single color. Applications were shown towards imaging of Cav1 and Dyn2K44A within the macromolecular complex of caveolae. It was demonstrated that the method provided the resolution to afford insight into the nanoscopic geometry of their organization.

In future, our imaging method could be combined with the recent DNA PAINT technique [51] in order to further improve the available resolution. With the use of the transient binding of short fluorescently labeled oligonucleotides to nanobodies, the imaging could be performed as long as it is needed to accumulate the sufficient number of fits with very small localization errors. This would allow to access cellular nanoscale geometry that has previously been limited by the available resolution resulting from localization fits of a limited number of fluorescent labels on the nanobodies.

The established dual color imaging approach was further extended to a biplane detection scheme to provide a novel two-color 3D superresolution technique. Imaging was demonstrated with a resolution typically shown for single-color experiments, while again eliminating many obstacles inherent to multicolor acquisition, which are even more serious in 3D.

In principle, spectral-demixing approach can be used with other combinations of spectrally close fluorophores. Two-dimentional three-color spectral-demixing FPALM has been already shown with the use of Dendra2, PAmCherry1, and PAmKate [33]. This could allow our 3D approach to be performed in live cells.

The developed labeling and imaging strategies were used for the detailed structural study of filamentous cytoskeletal structures assembled from mammalian septins. According to the current model, in the first stage of polymerization, mammalian septins self-assemble into hexameric ~27 nm long or octameric ~35 nm long rod-shaped complexes. In the next stage, these complexes anneal end-to-end into protofilaments, which in turn form various higher-order structures: filaments, rings, gauzes. (The assembly of individual septin complexes into filaments in
mammalian cells has never been studied \textit{in vivo} before.)

The genome-edited cell-line, where all endogenous SEPT2 was GFP-tagged, in combination with the established nanobody-staining strategy, provided a perfect tool for SMLM imaging septins with maximized labeling efficiency and in their native environment. The resolution below 10 nm achievable by SMLM methods in combination with advanced image analysis was used to find systematic patterns and rules for septin assembly in membranous filaments. The superresolved central SEPT2-dimer within the complex appeared as chains of localizations along filaments with regular spacing, suggesting the end-to-end assembly of septin complexes in filaments underlying the membrane of interphase mammalian cells. This result shows that \textit{in vivo} septin complexes assemble end-to-end into strands in accordance with \textit{in vivo} studies.

Additionally, it was shown that individual filaments exhibited a periodicity in SEPT2, though with variations in the distance between SEPT2 molecules. We observed equidistant spacings of 30-40 nm, 50-60 nm and 70-80 nm between SEPT2 molecules in different filaments. These periodicities provide information about the length of septin complexes that comprise filaments in cells. The observed larger periods are not in a full agreement with the current model of hexameric and octameric complex composition and show that septin complex composition is more complicated. Considering the number of septin homologs that could be expressed in mammalian cells, one can speculate that larger and more complicated complex assemblies may be possible (Figure 8.3). Using gene knockdown methods in combination with the imaging technique established in this study, would allow for further study into the contribution of individual septins in the composition and length of septin complexes.

The absolute arrangement of septins within complexes clearly requires the precise determination of the spatial relationship between different septin monomers. Here, two-color imaging of SEPT2 in the center and SEPT7 flanking the ends of hexameric complex showed that SEPT2 and SEPT7 were alternating in a seemingly regular fashion along filaments, confirming an end-to-end assembly of septin complexes. Further, we detected a distance of $\sim 8$ nm between SEPT2 and SEPT7 with spatial cross-correlation analysis. This is in good agreement with the structure of both hexameric and octameric complexes. These results suggest that in
**SUMMARY AND OUTLOOK**

**Septin subunits**

<table>
<thead>
<tr>
<th>SEPT2 group</th>
<th>SEPT3 group</th>
<th>SEPT7 group</th>
<th>SEPT6 group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 4 5</td>
<td>7 1 2</td>
<td>2</td>
<td>4 8 10 11</td>
</tr>
</tbody>
</table>

**Hetero-oligomeric complexes**

- **Canonical complexes**
  - Sirajuddin et al., 2007
  - Kim et al., 2011
  - Sellin et al., 2014

- **This study**
  - up to 70nm

**Filaments**

- **Canonical model**
  - up to 70nm

- **This study**
  - up to 70nm

Figure 8.3: **Model of mammalian septin complex and filament assembly.** Septin subunits are classified into four homology groups (shown in different colors). Septins from different groups form heteromeric and octameric complexes that assemble end-to-end into filaments. The results obtained in this study suggest larger complex assemblies than the canonical model.
some cases the additional septins anneal at the ends of the canonical septin complexes, which leads to larger distances between SEPT2-dimers observed (Figure 8.3). Furthermore, these results rule out that additional septins are inserted between SEPT2 and SEPT7. In the future, the establishment of an efficient and close labeling strategy for a number of endogenous septins could facilitate the detailed structural study of septin complexes composition \textit{in vivo} by the two color SMLM.

The exact composition and size of mammalian septin complexes may depend on the association of the filaments to different cellular elements, e.g. the plasma membrane, microtubule cytoskeleton or F-actin. Two color SMLM investigation of septins and filamentous actin revealed, that all septin assemblies studied in this work were associated to actin meshes underlying the basal membrane. In the future a detailed structural study of septin assemblies in context of their interaction partners, such as myosin II and Cdc42, will facilitate the understanding of principles governing the observed variety in the periodicities of SEPT2 molecules along different filaments.

The developed imaging and labeling methods were further used to resolve the dynamics and organization of septins on the plasma membrane. It is not yet fully understood how septins from the cytosol are incorporated into membrane-associated filamentous assemblies in live cells. When the canonical model of octameric and hexameric complexes suggests that septins exchange exclusively as complexes, it was also proposed that septins exchange within filaments as monomers [35] or as a pair of complexes [16]. In order to determine the minimal unit of exchange within septin filaments, we employed stepwise bleaching method together with an advanced automated analysis. The genome-edited cell-line, where all endogenous SEPT2 was GFP-tagged, provided a perfect tool for studying septin assemblies in their native environment. It was established in live-cell experiments that septins newly incorporated into membrane-bound filaments contained a SEPT2-SEPT2 dimer. This stoichiometry was further supported by \textit{in vitro} pull-down essays combined with stepwise bleaching using whole-cell lysate. Further pull-down experiments on the septin complex end-capping subunit SEPT9 showed that the observed SEPT2 dimers are in assembled octameric complexes.

Our investigation of the dynamics of membranous septins further by single-
molecule localization and live-cell single-particle tracking revealed new aspects of their assembly. The experiments showed that complexes diffuse freely in the range of few hundreds of nanometers followed by their incorporation into filaments. Thanks to the combination of live-cell imaging of endogenous septins with sub-diffraction resolution, moreover, it could be shown that complexes, as opposed to previous studies [8], can incorporate over the entire length of preexisting filamentous septins (Figure 8.3).

Figure 8.4: Suggested mechanism of mammalian cytoskeleton dynamics according to the results in this study and in context of literature.
In future, the assembly and dynamics can be further investigated by use of other advanced single and multi color live-cell superresolution method, such as SIM [34], SOFI [18] or 3B [14]. This would allow deepening our understanding of the rules that govern the assembly and disassembly of septin higher-order structures and the diffusion of septin complexes in a context of other cytoskeletal components and septin-interaction molecules.

In conclusion, new advancements were brought to the field of superresolution microscopy and new insights were gained into the assembly of the fourth component of the cytoskeleton - the septins.
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