Review

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Imaging the invisible: resolving cellular microcompartments by superresolution microscopy techniques

Abstract: Unraveling the spatio-temporal organization of dynamic cellular microcompartments requires live cell imaging techniques capable of resolving submicroscopic structures. While the resolution of traditional far-field fluorescence imaging techniques is limited by the diffraction barrier, several fluorescence-based microscopy techniques providing sub-100 nm resolution have become available during the past decade. Here, we briefly introduce the optical principles of these techniques and compare their capabilities and limitations with respect to spatial and temporal resolution as well as live cell capabilities. Moreover, we summarize how these techniques contributed to a better understanding of plasma membrane microdomains, the dynamic nanoscale organization of neuronal synapses and the sub-compartmentation of microorganisms. Based on these applications, we highlight complementarity of these techniques and their potential to address specific challenges in the context of dynamic cellular microcompartments, as well as the perspectives to overcome current limitations of these methods.

Keywords: cellular microcompartment; nanoscopy; near-field scanning optical microscopy; single molecule localization; stimulated emission depletion; superresolution imaging.

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Introduction

Cellular microcompartments comprise proteins, lipids, nucleic acids and other components, which are organized on a submicroscopic level in a spatially and temporally well-defined manner. Unraveling the functional organization of cellular microcompartments by microscopic techniques is highly challenging. Electron microscopy is capable of revealing the morphology of cellular structures with the highest resolution. Recent developments in cryo-EM tomography have been able to identify individual multi-protein complexes within whole cells (Leis et al., 2009). However, the precise localization and spatial arrangement of specific molecules within these structures remains difficult. Here, the key problem is to achieve exogenous labeling with electron-dense markers with high efficiency while preserving cellular ultrastructure. More fundamentally, the spatio-temporal dynamics of proteins cannot be captured by EM techniques, which require fixation of the sample prior to imaging. Imaging of proteins by fluorescence microscopy has contributed enormously to unravel the localization, mobility and interactions of proteins in living cells. A broad variety of fluorescent proteins with different spectral and photophysical properties is available for genetically encoded labeling target proteins. Moreover, various fusion proteins for selective posttranslational labeling in living cells are available, which further extend the repertoire of fluorescence dyes (Johnsson and Johnsson, 2007). Owing to the wavelength-dependent diffraction barrier, however, the resolution of conventional, far-field fluorescence imaging techniques is limited to 200–300 nm and 500–800 nm in lateral and axial dimensions, respectively. For this reason, nanoscopic protein organization within cellular microcompartments cannot be resolved by conventional far-field fluorescence microscopy.

Here, we will briefly introduce recent developments in fluorescence imaging, which succeeded to overcome the diffraction barrier, and compare their capabilities and limitations for unraveling the dynamic organization of cellular microcompartments. We will review how these techniques have already contributed to gaining novel insight into the functional compartmentalization of signaling complexes in the plasma membrane, neuronal synapses and microorganisms.
Near-field imaging techniques

The first fluorescence imaging technique yielding submicroscopic resolution exploited the optical near-field, which is not limited by diffraction. For near-field scanning optical microscopy (NSOM), fluorescence excitation at the tip of an aperture with a diameter of a few 10 nm is scanned across the sample (Figure 1A) (de Lange et al., 2001). Because of the submicroscopic dimension of the aperture, light cannot propagate into the sample and thus fluorescence excitation is limited to the evanescent field at the surface of the tip, the optical near-field. By this technique, a lateral resolution of ~50 nm has been achieved, which is determined by the size of the aperture of the illuminating tip and its distance from the sample. In order to ensure optimum illumination, the distance between the aperture and the sample needs to be controlled, which is typically achieved by a shear force feedback loop. Thus, not only the fluorescence is recorded with submicroscopic resolution, but also the topography of the sample. While the penetration depth of the optical near-field into the sample is in the range of ~100 nm, the topography of the sample can be resolved with an axial resolution of a few nanometers. These features make NSOM a powerful technique for probing the compartmentalization of the plasma membrane (van Zanten et al., 2010), but it cannot be applied for imaging intracellular structures. Moreover, NSOM is mostly applied to fixed cells because of the need to bring the aperture in close proximity and to scan it over the sample with relatively slow speed. Yet, NSOM offers means to probe the diffusion dynamics of protein and lipids in the plasma membrane in submicroscopic dimensions by using the optical near-field for fluorescence correlation spectroscopy (FCS) (Huang et al., 2010). Some of these methods – such as structured illumination microscopy (SIM) (Schermelleh et al., 2008; Kner et al., 2009), or dynamic saturation optical microscopy (DSOM) (Humpolickova et al., 2010) – have achieved an increase in the resolution by a factor of 2–3. While subcellular structures can be captured in significantly more detail and less background by these techniques, features below 100 nm cannot be resolved. A resolution significantly below 100 nm has been achieved by techniques that are fundamentally based on the possibility of switching fluorophores between a fluorescent and a non-fluorescent state (Hell, 2009). Two conceptually different strategies have been implemented, which employ either targeted or stochastic switching and readout (Hell, 2007, 2009; Patterson et al., 2010; Toomre and Bewersdorf, 2010; Gould et al., 2012; Moerner, 2012).

Far-field imaging techniques

During the past decade, far-field fluorescence imaging techniques have also succeeded to overcome the diffraction barrier by exploiting specific properties of fluorescence dyes (Huang et al., 2010). Some of these methods – such as structured illumination microscopy (SIM) (Schermelleh et al., 2008; Kner et al., 2009), or dynamic saturation optical microscopy (DSOM) (Humpolickova et al., 2010) – have achieved an increase in the resolution by a factor of 2–3. While subcellular structures can be captured in significantly more detail and less background by these techniques, features below 100 nm cannot be resolved. A resolution significantly below 100 nm has been achieved by techniques that are fundamentally based on the possibility of switching fluorophores between a fluorescent and a non-fluorescent state (Hell, 2009). Two conceptually different strategies have been implemented, which employ either targeted or stochastic switching and readout (Hell, 2007, 2009; Patterson et al., 2010; Toomre and Bewersdorf, 2010; Gould et al., 2012; Moerner, 2012).

Targeted switching and readout

Targeted readout superresolution imaging techniques are based on limiting the dimension of the effective area, which has been achieved by stimulated emission depletion (STED)
(Hell and Wichmann, 1994; Klar et al., 2000), reversible saturable optical fluorescence transitions (RESOLFT) (Hofmann et al., 2005) and ground state depletion (GSD) (Hell and Kroug, 1995). STED microscopy is based on reducing the diffraction-limited volume of spontaneous fluorescence emission by spatially selective de-exciting fluorophores using stimulated emission (Figure 1B). This is achieved by an overlay of a focused excitation beam with STED beam featuring a central zero (i.e., a doughnut shape). Thus, fluorescence emission is effectively limited to the center of excitation volume. By scanning the sample, images with a resolution of 40 nm and below can be obtained. This technique has been very successfully applied for resolving protein organization in cellular microcompartments, such as dendritic spines (Nagerl et al., 2008) and synapses (Kittel et al., 2006) as well as plasma membrane microdomains (van den Bogaart et al., 2011). STED is in principle possible with every fluorescence dye but the imaging conditions have to be adjusted carefully to their photophysical properties. Live cell imaging has been achieved with fluorescent proteins (Hein et al., 2008), yet posttranslational labeling with fluorescence dyes more suitable for STED appears to be particularly promising (Hein et al., 2010; Lukinavicius et al., 2013). For RESOLFT and GSD the same illumination scheme as STED is employed, but specific properties of some fluorescence dyes that can be reversibly switched off by light are exploited for these techniques, which allow imaging at reduced light levels (Testa et al., 2012).

The microscopic set-up for STED microscopy is technically rather demanding and multicolor imaging remains challenging. Further challenges arise from potentially high photobleaching and toxicity, as well as relatively long acquisition times. However, commercial STED microscopes are available and recent technical developments using continuous wave laser excitation and time-gated detection promise further simplification of the experimental set-up and reduction of phototoxicity (Willig et al., 2007; Vicidomini et al., 2011). Because of its targeted readout, STED provides a unique means for probing fast processes with submicroscopic resolution (Westphal et al., 2008). Moreover, STED opens exciting possibilities for probing diffusion and interaction of molecules in cellular microcompartments by FCS with sub-diffraction resolution (STED-FCS) (Kastrup et al., 2005; Eggeling et al., 2009).

Single molecule localization-based imaging

As an alternative to a targeted readout, stochastic switching of fluorophores provides means for superresolution and has been successfully employed for imaging with a resolution significantly below 100 nm, either based on the localization of individual fluorescent emitters (Patterson et al., 2010; Moerner, 2012) or by spatial and temporal correlation of single molecule fluctuations (superresolution optical fluctuation imaging, SOFI) (Dertinger et al., 2010). SOFI is an elegant approach not only for increasing the resolution of fluorescence images, but also for efficiently reducing the background fluorescence. This technique, however, requires fixed samples in order to avoid fluctuations caused by diffusion, and thus cannot capture the dynamics of cellular microcompartments. In contrast, single molecule localization-based superresolution imaging techniques have provide powerful means to resolve microcompartments in both fixed and live cells. The basic concept of these techniques is summarized in Figure 2: by determining the center of gravity of their

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**Figure 2** Principles of single molecule localization-based superresolution imaging techniques. (A) Single molecule localization beyond the diffraction limit by fitting the point spread function of individual molecules, e.g., by a Gaussian function (blue line in the right panel). The localization precision illustrated by the red line is proportional to the square root of the number of photons detected for each molecule (Gould et al., 2012). (B) The super-resolved image is obtained by localizing individual molecules within a stack of >1000 frames, each capturing a different subset of individual molecules from the total ensemble. (C) Irreversible photoactivation or photoswitching as used for (F)PALM. (D) Reversible photoswitching as applied for STORM and related techniques.
point spread function (Figure 2A), individual molecules can be localized with a precision of a few nanometers, mainly depending on the number of collected photons. Single molecule localization has been previously applied (Schmidt et al., 1996; Thompson et al., 2002), e.g., for tracking individual molecules in vitro and in living cells. Its application to obtaining images of dense protein ensembles, which cannot be simultaneously imaged on the single molecule level, requires stochastic activation of a subset of fluorescent probes. Thus, the density of emitting probes in each frame can be limited to a number, which can be resolved by diffraction-limited imaging. By sequentially imaging stochastically activated subsets, a cumulative image of the ensemble can be rendered from the molecules localized in each frame (Figure 2B). As the image is composed of dots representing detected molecules, these techniques have been referred to as ‘pointillism’. Various means for stochastic activation of probes have been employed for this approach, prominently by using photoactivatable (PA) or photoswitchable (PS) fluorescence probes (Betzig et al., 2006; Hess et al., 2006), or with photoswitchable synthetic dyes (Rust et al., 2006).

With photoactivatable fluorescent proteins becoming available (Lippincott-Schwartz and Patterson, 2009), these were successfully employed for (fluorescence) photoactivation localization microscopy (PALM/FPALM) (Betzig et al., 2006; Hess et al., 2006). The principle of these techniques is depicted in Figure 2C: after photoactivation, molecules are rapidly photobleached during readout and continuously replenished by photoactivation of further subsets until the entire ensemble has been probed. The typical lateral resolution obtained by this approach is <40 nm, mainly depending on the number of photons collected for each molecule and the background fluorescence. Because fluorophores are switched off by photobleaching, each molecule of the ensemble can be detected only once. By using reversibly photoswitchable (photochromic) fluorescent proteins, the possibility for multiple readout of each fluorophore has been demonstrated (Flors et al., 2007). (F)PALM is readily applicable in live cell imaging as a broad choice of genetically encoded photoactivatable and photoswitchable probes are available. Dual- (Shroff et al., 2007) and triple-color (Gunwardene et al., 2011) imaging has meanwhile been achieved, and the palette of photoconvertible fluorescent proteins is constantly expanding. Moreover, the diffusion dynamics of proteins can be readily probed by combination with single molecule tracking (Hess et al., 2007; Manley et al., 2008).

Stochastic optical reconstruction microscopy (STORM) is based on labeling with photoswitchable synthetic dyes (Rust et al., 2006). This technique exploits the property of certain fluorophores to enter into a relatively stable (milliseconds-seconds) dark state upon illumination, which can be caused by different photochemical processes (Ha and Tinnefeld, 2012). After switching off the majority of fluorophores by the readout-laser, subensembles are recovered by photoactivation in a stochastic manner and switched off again during readout (Figure 2D). In conventional STORM, photoactivation is achieved by energy transfer from another dye in close proximity (photochromic blinking), which provides flexible means for multicolor imaging (Bates et al., 2007). Meanwhile, reversible photoswitching in the absence of a sensitizing fluorophore as well as spontaneous return into the ground state has been demonstrated (direct STORM, dSTORM or ground state depletion microscopy followed by individual molecule return, GSDIM), thus simplifying the acquisition process substantially (Folling et al., 2008; Heilemann et al., 2008; van de Linde et al., 2013). As a broad range of fluorophores is compatible with these approaches (Heilemann et al., 2008, 2009; Dempsey et al., 2011), multicolor superresolution imaging is readily implemented (Bates et al., 2007; Bossi et al., 2008; Testa et al., 2010).

Reversible photoswitching requires a strongly reducing environment of the fluorophore in order to avoid irreversible photodestruction by reaction with oxygen. For this reason and because of the requirement to introduce labels exogenously, STORM is mostly performed with fixed cells. However, some fluorophores show reversible photoswitching in the cytosol (Heilemann et al., 2009). By selectively targeting these dyes to proteins in live cells by posttranslational labeling techniques (Johnsson and Johnsson, 2007) dSTORM imaging of intracellular microcompartments is possible in living cells (Wombacher et al., 2010). Because of reversible photoswitching, dSTORM in living cells can be employed for capturing the dynamics of cellular nanostructures (Testa et al., 2010; Wombacher et al., 2010; Shim et al., 2012; Wilmes et al., 2012; van de Linde et al., 2013). (F)PALM and (d)STORM have very similar requirements in terms of laser illumination and detection capabilities of the microscope. Combination of both techniques is readily possible and has been successfully employed for implementing triple-color superresolution imaging in living cells (Wilmes et al., 2012). The repertoire of fluorescence dyes amenable for single molecule localization microscopy techniques is constantly expanding as many fluorescence dyes including autofluorescent proteins photobleach at high-power excitation (Biteen et al., 2008; Testa et al., 2010; Burnette et al., 2011; Cox et al., 2012). However, fast and exhaustive multicolor imaging of protein ensembles with high resolution in living cells requires highly specific blinking properties.
and high photon yields. For fully exploiting the potential of (d)STORM and (F)PALM techniques, further, systematic development of dyes and fluorescent proteins will be key.

In contrast to electron microscopy, fluorescence imaging techniques selectively visualize labeled species. In order to obtain morphological information in nanoscopic dimensions, a high density of fluorescence marker is necessary in order to obtain a sampling density that is sufficient according to the Nyquist criterion. Thus, high overexpression of marker proteins is required, which may bias assembly and dynamics of cellular microcompartments. In case of mobile marker such as membrane proteins, the morphology of structures can also be captured by following a small population of molecules while they diffuse within nanoscopic structures (Figure 2C). Instead of rendering an image from multiple subpopulations of a large ensemble, it is obtained from a small population of molecules constantly changing their position within the cellular structure. This technique, called tracking and localization microscopy (TALM), not only captures the morphology of microcompartments, but also the connectivity and the dynamics of protein within these structures (Appelhans et al., 2012). TALM, however, critically depends on the photostability of the fluorescent probe, and therefore has been successfully introduced by using posttranslational labeling with a synthetic dye (Appelhans et al., 2012). While the time resolution of (F)PALM and (d)STORM are mainly limited by the acquisition rate, for TALM the mobility of the probe also plays a critical role. Diffusion constants of proteins in organellar membranes (0.1–1 μm²/s) are well suitable for TALM, as demonstrated for the outer and the inner mitochondrial membranes (Appelhans et al., 2012). In case of relatively fast diffusing probes (D>0.1 μm²/s), the position of the molecule varies substantially within the acquisition time. Thus, the effective spatial resolution can be increased by more rapid acquisition or pulsed excitation.

While single molecule localization techniques were originally applied to increasing the lateral resolution, the axial resolution can also be substantially enhanced. 3D superresolution imaging by (F)PALM was initially achieved by simultaneous fluorescence detection in two focal planes of the sample (biplane imaging), yielding an axial resolution of ~80 nm (Juette et al., 2008). More robust 3D localization and higher axial precision have been achieved by an asymmetric distortion of the point spread function dependent on the axial position employed. By using optical astigmatism, 3D STORM imaging of a whole cell with an axial resolution <70 nm was achieved (Huang et al., 2008a,b). Further improvement of the 3D resolution of single molecule localization techniques has been achieved by a double helical point spread function (Pavaniet al., 2009). By using interferometric approaches requiring simultaneous illumination and detection through two objectives, 3D localization below 20 nm has been reported for PALM (Shtengel et al., 2009) and STORM (Aquino et al., 2011; Xu et al., 2012).

### Signaling microcompartments in the plasma membrane

In multicellular organisms, cells are confronted with an enormous variety of stimuli, which need to be integrated for making appropriate decisions in the context with their own homeostasis. These stimuli are recognized and processed by proteins in the plasma membrane – receptors, ion channels, transporters and enzymes. The functional organization of these proteins within the plasma membrane remains obscure, yet an enormous complexity in their spatio-temporal organization is emerging. With their capability to probe the diffusion dynamics of proteins and lipids, early fluorescence imaging techniques questioned the validity of the classic fluid mosaic model (Singer and Nicolson, 1972) for the mammalian plasma membrane. In particular single molecule and single particle tracking experiments revealed highly inhomogeneous diffusion properties, suggesting a more complex organization on submicroscopic scale. The cellular and physicochemical bases underlying the submicroscopic organization of the plasma membrane remain debated, but several contributing principles have clearly emerged: (i) lipid phase separation into nanoscopic, highly dynamic liquid-ordered domains, so-called ‘membrane rafts’; (ii) corral-like defined by the cortical actin skeleton, referred to as the ‘membrane skeleton’; (iii) interactions with scaffold proteins and the formation of multi-protein complexes (Kusumi et al., 2005; Lillemoier et al., 2006; Marguet et al., 2006; Lingwood and Simons, 2010). It has been suggested that these principles contribute in a hierarchical manner to the formation of diverse plasma membrane microcompartments (Kusumi et al., 2010) with their properties depending on the protein and lipid composition. A key challenge for the functional characterization of these submicroscopic plasma membrane microcompartments is their highly dynamic assembly and disassembly caused by rapid exchange of proteins and lipids with the environment. While single particle tracking techniques are powerful tools to map diffusion properties in the plasma membrane with very high spatial and temporal resolution (Serge et al., 2008), they only indirectly provide information about the underlying structures. In contrast,
sub-diffraction fluorescence imaging techniques have the potential simultaneously capture the morphology of plasma membrane microcompartments and the dynamics of biomolecules within these structures.

As NSOM is compatible for imaging the plasma membrane, it was the first fluorescence technique to directly resolve submicroscopic protein clusters (Hwang et al., 1998; Nagy et al., 1999). By combination with quantum dot labeling, organization of the T-cell receptor on the surface of T-cells was observed with a resolution <50 nm (Chen et al., 2008b). Dual-color NSOM imaging for the first time clearly resolved the localization of proteins (de Bakker et al., 2008; van Zanten et al., 2009; Zhong et al., 2009) and lipids (Chen et al., 2008a) in different types of domains (Figure 3A,B). These results, however, were obtained with fixed samples and thus the spatio-temporal dynamics of these domains could not be resolved. While all these studies were performed with fixed cells, NSOM was recently employed for probing lipid diffusion in the plasma membrane by FCS with sub-diffraction resolution, revealing differential local diffusion properties of phosphatidylcholine and sphingomyelin (Manzo et al., 2011).

Similarly, clustering of receptors in the plasma membrane has been resolved by STED microscopy (Kellner et al., 2007; Sieber et al., 2007). More importantly, however, nanoscale heterogeneity in the diffusion dynamics of raft lipids in the plasma membrane has been studied in unprecedented detail by STED-FCS (Eggeling et al., 2009; Mueller et al., 2011). As the spot size for FCS could be reduced down to a diameter of 30 nm, lipid diffusion could be probed on a length scale as predicted for membrane rafts. Comparison of different lipids revealed that rather than a change of viscosity, transient binding events were responsible for heterogeneous diffusion of typical raft lipids, suggesting that lipid-protein interactions play a critical role for the spatio-temporal organization of these lipids. These studies are based on a systematic variation of the spot size, which can be very elegantly achieved by time-gated STED (Vicidomini et al., 2011). However, STED imaging in combination with STED-FCS is required in order to understand the nanoscale diffusion dynamics in the context of cellular nanostuctures, which has not yet been reported yet potentially could be resolved by scanning FCS (Ruan et al., 2004).

Submicroscopic protein organization in the plasma membrane has also been resolved by single molecule localization techniques such as (F)PALM (Subach et al., 2009; Owen et al., 2010) and STORM (Jones et al., 2011). With its capability for multicolor detection, spatial organization of proteins within clathrin-coated pits or the membrane skeleton could be resolved (Subach et al., 2009; Owen et al., 2010; Jones et al., 2011; Wilmes et al., 2012) (Figure 3C–F). PALM proved powerful for the quantitative characterization of protein clustering in fixed cells, because each molecule is detected and localized separately. Thus, organization of individual proteins within clusters, clusters size distribution and their numbers can be quantified by spatial correlation of individual proteins (Sengupta et al., 2011). This technique, termed pair-correlation PALM (PC-PALM), was able to discriminate defined protein oligomerization from microdomain-dependent clustering and to quantitatively assess the submicroscopic organization of GPI-anchored in membrane rafts (Sengupta et al., 2011).

In contrast to NSOM and STED, diffusion properties and morphological information on a submicroscopic scale is readily obtained simultaneously by single molecule localization techniques. As individual molecules are being detected, their local diffusion properties can be analysed by tracking their position frame-by-frame until these molecules are bleached. Soon after establishing (F)PALM, combination with single molecule tracking was successfully applied to explore the dynamic clustering of proteins in the plasma membrane on a submicroscopic scale (Hess et al., 2007). These studies revealed the existence of dynamic microcompartments on different-length scales, which appeared to be organized by scaffold structures – probably the membrane skeleton. This technique has been termed single particle tracking PALM (sptPALM), and was readily extended to dual-color imaging (Subach et al., 2010) (Figure 3D). The combination of single molecule tracking and localization microscopy techniques, as employed for sptPALM or TALM, holds great promises for unraveling the spatio-temporal organization of the plasma membrane. These techniques profit greatly from newly developed fluorescent dyes and photoswitchable fluorescent proteins, which continue to enhance multiplexing capabilities and observation times. Yet, a key limitation for the application of these techniques for unraveling the spatio-temporal dynamics of plasma membrane microcompartments is the relatively long time-span required for obtaining morphologic information. These submicroscopic structures are believed to fluctuate on a sub-second time-scale. Even most rapid image acquisition by new, highly sensitive sCMOS cameras (Long et al., 2012) need >1 s to provide 1000 frames as required for rendering meaningful superresolution images.

**Compartmentalization of neuronal synapses**

In the central nervous system, communication between nerve cells occurs at specialized junctions called synapses.
At the chemical synapse, electrical signals trigger controlled secretion of neurotransmitter via exocytosis of synaptic vesicles (SV) from the presynaptic nerve terminal. The fusion of SVs releases neurotransmitters that diffuse across the synaptic cleft and activate receptor channels in the receiving neuron at the so-called ‘postsynaptic site’. That generates a new electric signal in the postsynaptic cell (Katz and Miledi, 1965, 1967). Given the limited number of SVs in a presynaptic terminal, complete fusion of many SVs with the plasma membrane would result in fast depletion of fusion fusion-competent SVs and thereby the loss of the ability to transmit signals across synapses. Therefore, to support rapid and repeated rounds of release, a fast trafficking cycle is required to maintain synaptic function during sustained synaptic activities (Harata et al., 2001; Sudhof, 2004; Schweizer and Ryan, 2006). Although intense research over several decades has led to a deep understanding of the molecular mechanisms of exocytic membrane fusion (Jahn and Scheller, 2006; Martens and McMahon, 2008) and endocytic retrieval of SV (Dittman and Ryan, 2009; Doherty and McMahon, 2009), comparatively little is known about the coupling between exo- and

**Figure 3** Unraveling compartmentalization of the plasma membrane by fluorescence imaging techniques. (A, B) Clustering of DC-SIGN in immature dendritic cells revealed by NSOM imaging (B) compared to traditional confocal microscopy (A) (from de Bakker et al., 2007, with permission). (C, D) Submicroscopic dynamic organization of vesicular stomatitis virus G protein (green) and epidermal growth factor receptor (red) in the plasma membrane resolved sptPALM (from Subach et al., 2010, with permission). (C) Cumulative FPALM image. (D) Single molecule trajectories. (E, F) Organization of the type I interferon receptor subunits IFNAR1 (green) and IFNAR2 (red) in context of the membrane skeleton (blue) as resolved by FPALM and dSTORM in living cells (from Wilmes et al., 2012, with permission).
endocytosis. Hence, understanding the spatial organization of exo- and endocytosis in putatively scaffold-based nanodomains should help to unravel the interplay and molecular coupling mechanism of exo- and endocytosis. To this end the application of the above-described super-resolution microscopy techniques have made tremendous inroads in understanding the spatial organization of synaptic transmission, synaptic homeostasis, and plasticity.

The presynaptic terminal contains clusters of SVs, key organelles of chemical neurotransmission. Proteomics and lipidomic data indicate that SVs comprise distinct sets of proteins and lipids present in defined stoichiometries (Takamori et al., 2006), which must be maintained during repetitive rounds of exo- and endocytosis. Most synaptic vesicle proteins are present in surprisingly low copy numbers per vesicle. In some cases [such as the synaptic vesicle 2-related protein (SV2) or the vacuolar ATPase that provides the proton-motive force for filling SVs with about 1800 transmitter molecules], even just one or two are present per vesicle. How this precise sorting of SV membrane proteins is accomplished molecularly is not understood. Initially, STED microscopy suggested that SV components could remain clustered, thereby tightly coupling exo- and endocytosis (Willig et al., 2006) during their exo-endocytic itinerary. However, more recently dual-color 3D STED, so-called isoSTED (Schmidt et al., 2008) with a resolution of 40 nm in all dimensions, combined with physiological analysis rather points to a mechanism in which SV components are resorted and reclustered at the cell surface (Wienisch and Klingauf, 2006; Hua et al., 2011) by individual sorting via recognition of sorting determinants by cargo-specific adaptor proteins such as AP-1, AP-2, stonin2/stoned B (Jung et al., 2007; Glyvuk et al., 2010). SV exo- and endocytosis appear to be temporally coupled but spatially segregated between the active zone (sites of fusion) and the surrounding endocytic or periactive zone (Wienisch and Klingauf, 2006). The tight temporal coupling between SV exo- and endocytosis suggests that SV cargo protein sorting and recycling likely involves precise control of the localization and dynamics of proteins or protein complexes as they partition between sites of fusion (active zone) and endocytosis (periactive zone, Figure 4) (Augustine et al., 2003; Hua et al., 2011).

The release sites within the active zone are thought to be characterized by the spatial proximity of docked vesicles (also so-called readily releasable) and presynaptic Ca\(^{2+}\) channels (Augustine et al., 2003). STED, genetic and morphological data from Drosophila melanogaster neuromuscular junction showed that such geometrical arrangements involve giant scaffold proteins such as Rab3-interacting molecules (RIMs), piccolo, bassoon and bruchpilot (BRP) (Fouquet et al., 2009), which are large enough to bridge multiple presynaptic microdomains over distances of up to several hundred nanometers. By associating with exocytic–endocytic proteins, with components of the actin cytoskeleton such as actin binding protein 1 (ABP1) and profilin (Fenster et al., 2003; Connert et al., 2006), and with each other, these factors may assemble into a matrix that provides attachment sites for the spatiotemporally directed movement of synaptic

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**Figure 4** Submicroscopic organization of cultured hippocampal synapses. Three-dimensional dual-color isoSTED microscopy shows the localization of surface-stranded SV protein synaptotagmin 1 (Syt1) (red) around the active zone visualized by the opposing post-synaptic density scaffolding protein Homer1 (green). (A) z-projection of a three-dimensional image stack (from Hua et al., 2011, with permission). Scale bar 1 μm. (B–D) Perspective views of volume-rendered data. The Syt1 labeling reveals a few preassembled patches (‘readily retrievable pool’ of SVs), localized in the periphery of the active zone.
vesicle membranes between functionally distinct membrane domains.

Genetic analysis of presynaptic scaffolds has also turned out to be difficult because of the complex (broad) phenotypes resulting from null alleles. For example, deletion of the active zone component BRP in Drosophila melanogaster causes a severe loss of active zone dense bodies (T-bars; appear T-shaped in EM pictures) and a concomitant declustering of presynaptic Ca\(^{2+}\) channels, provoking a severe deficit in baseline synaptic vesicle release (Kittel et al., 2006). A recent study (Hallermann et al., 2010) identified a hypomorphic allele, brp\textsuperscript{pabe}, lacking merely the last 17 amino acids of BRP. In brp\textsuperscript{nude} flies, electron-dense T-bars representing the cytomatrix of the active zone were properly shaped, but completely lacked the SVs they are normally studded with. Although basal glutamate release was unchanged, paired-pulse stimulation provoked a severe depression. Furthermore, rapid recovery following sustained release was slowed down (Hallermann et al., 2010). A recent two-color STED microscopy study (Liu et al., 2011) uncovered yet another protein, the Drosophila RIM-binding protein (DRBP), as essential not only for the integrity of the cytomatrix at the active zone but also for exocytotic neurotransmitter release. DRBP surrounds the central Ca\(^{2+}\) channel field. In loss of function mutants, Ca\(^{2+}\) channel clustering and Ca\(^{2+}\) influx were impaired, and synaptic release probability was drastically reduced. Both data sets identify the BRP and RBP family proteins as prime effectors of the active zone scaffold that are essential for coupling of SVs, Ca\(^{2+}\) channels, the SV fusion machinery, and likely exocytotic–endocytic coupling.

Clearly, the analysis of active zone components and their functional relationship with exocytic–endocytic coupling at different types of synapses is far from complete. Combined genetic, physiological, biochemical and especially high resolution imaging approaches, will further expand our understanding of how presynaptic active zone microcompartments may direct and orchestrate synaptic vesicle cycling. As described above, several steps in the synaptic vesicle cycle necessitate a tight spatio-temporal coordination in defined microcompartments:

- Delivery of SVs to the release sites must be efficient to meet the high demand of release-ready SVs during periods of intense synaptic activity.
- Precise timing of transmitter release relative to the action potentials (‘synchronous release’) requires close proximity between Ca\(^{2+}\) channels and release-ready vesicles.
- High release rates of SVs at the release sites requires an effective mechanism of release site clearance in order to make room for newly arriving SVs and also to preserve the integrity of the release site.
- Re-sorting of SVs components from those of the plasma membrane has to be achieved prior to the endocytic retrieval of SVs within the readily retrievable pool at the periactive zone (Figure 4).

All these tasks require a complex set of proteins, some of which serve as structural scaffolds for the spatio-temporal coordination of these processes.

An important future task will be not only to define the complete network of interactions, but also to visualize the dynamics of this microcompartment in live cells. First successful attempts in this direction have been made using live and up to video-rate STED microscopy, on both the pre- and postsynaptic compartments. In a first study (Westphal et al., 2008) video-rate (28 frames/s) far-field STED microscopy with a focal spot size of 62 nm enabled mapping of the movement and mobility of single SVs in living presynaptic boutons. This study was the first to demonstrate the ability of optical microscopy to investigate intracellular physiological processes on the nanoscale in real time (Westphal et al., 2008). The first use of a far-field optical technique with sub-diffraction resolution to non-invasively image activity-dependent morphological plasticity of postsynapses in organotypic brain slices at depths up to 120 \(\mu\)m was accomplished soon thereafter (Nagerl et al., 2008). Specifically, time-lapse STED imaging of dendritic spines of YFP-positive hippocampal neurons was used for quantification of morphological parameters, such as neck width and spine head shape, which are thought to play critical roles for the function and plasticity of synaptic connections. Now, this has been even accomplished in anesthetized heterozygous TgN (Thy1-EYFP) mice transgenically expressing EYFP (Berning et al., 2012). Recently, even the distribution and dynamics of actin within spines, could be resolved this way at a resolution of 60–80 nm in living organotypic brain slices, i.e., within functionally intact brain tissue (Urban et al., 2011). Similar results have been obtained with a RESOLFT imaging scheme using a novel reversibly switchable enhanced green fluorescent protein (rsEGFP), which can be photoswitched more than a thousand times, requiring light intensities reduced several orders of magnitude compared to standard STED imaging (Grotjohann et al., 2012; Testa et al., 2012). In 2012 superresolution PALM live cell imaging of postsynaptic AMPA receptor trafficking and molecular interactions was achieved (Hoze et al., 2012). Simultaneous tracking of thousands of individual postsynaptic receptors in live cells was possible, from which the strength of their molecular interaction at the sub-diffraction level in
hippocampal dendrites and spines could be estimated. It was shown that the high density of AMPARs in the post-synaptic density (PSD; the postsynaptic microcompartment opposing the presynaptic active zone in spines) is generated by physical interactions with an ensemble of cooperative membrane binding sites, provided by postsynaptic scaffold proteins, such as PSD 95, rather than simple molecular crowding or aggregation.

The emerging ability of both deterministic (STED) and stochastic (PALM) high resolution imaging techniques to visualize the structure of the microcompartments on both sides of the synapse in molecular detail and even follow their dynamics in live cells, will certainly have a high impact in shedding light on one of the most important aspects of brain function.

**Bacterial microcompartments**

The analysis of biological processes in bacterial cells imposes specific problems. Bacteria are single cell organisms with reduced complexity in their lifestyle, but often with complex metabolic features, responses to changing environments and interactions with other organisms, such as bacterial pathogens of eukaryotic hosts. The generic bacterial cell lacks compartmentation of cellular processes within membrane-bound organelles such as nuclei, mitochondria, Golgi, ER, etc. However, despite the lack of intracellular membrane compartments, bacterial cells show distinct subcellular organization principles. Work from recent years demonstrated that bacterial cells show polarity and possess a complex and dynamic cytoskeleton, as well as sophisticated transport machineries for protein secretion and translocation. The term 'microcompartments' was initially used for bacterial suborganellar assemblies, particularly the carboxysomes (Yeates et al., 2008). In a broader sense, we meanwhile understand complex assemblies of proteins and, in part, membrane lipids, as microcompartments with functions in assembling and shaping the bacterial cells, in signal recognition and response, or interaction with other cells.

The investigation of the supramolecular organization of proteins in bacterial cells is generally hampered by the small size of the cell. A cell of *Escherichia coli*, a rod-shaped bacterial model organism, is approximately 0.5–1×1–2 μm in size with a cell volume of 0.5–2 μm³ (1–2 fl). Conventional light microscopy only yields a limited resolution of organization pattern in bacterial cells, this is just 20 pixel for an *E. coli* cells given the diffraction limit of 200 nm for light microscopy. While the application of conventional light microscopy, electron microscopy and tomography initiated the understanding resulted in fundamental progress in understanding bacterial cell biology, important advances have been recently made by application of superresolution imaging techniques. A more detailed coverage of the applications can be found in recent reviews (Biteen and Moerner, 2010; Coltharp and Xiao, 2012).

Initial work focused on the organization and dynamics of the bacterial cytoskeleton. Bacterial cells possess a dynamic cytoskeleton consisting of filaments with tubulin-related FtsZ and actin-related MreB or ParM (reviewed in Graumann, 2007; Thanbichler and Shapiro, 2008). FtsZ proteins assemble into a ring structure that positions the division plane for binary fission of bacterial cells. The MreB filaments position cell wall assembly sites and by this determine cell shape. Par filaments are required for the segregation of chromosomal and episomal DNA between dividing cells. Further filaments, i.e., the intermediate filament-related crescentin, govern the specific cells shape of bacterial genera such as *Caulobacter* spp., *Vibrio* spp., or *Helicobacter* spp., or position organelles like magnetosomes through action of MamK.

Analyses of bacterial cytoskeletal elements were severely obstructed by diffraction-limited microscopy and superresolution microscopy provided major advances in the molecular analysis. By exploiting spontaneous photo switching of EYFP fused to MreB, imaging of dynamic MreB filaments in living cells with a resolution of 40 nm was possible (Biteen et al., 2008) (Figure 5A, B). An exceptionally high resolution of MreB filaments in living *E. coli* cells was obtained by the application of the RESOLFT approach (Grotjohann et al., 2012). Fu et al. (2010) investigated the FtsZ ring in living bacteria using PALM microscopy. The analyses resulted in the determination of the thickness of the FtsZ ring of 110 nm, indicating a discontinuous assembly of protofilaments, rather than a continuous FtsZ ring. The organization of the FtsZ ring was investigated using a 3D-SIM approach (Strauss et al., 2012). Previous studies proposed a rather static uniform FtsZ ring. In contrast, this work revealed a distribution of FtsZ subunits in discontinuous ring-shaped filaments and a bead-like distribution of cell wall-assembling divisome complexes along the FtsZ ring. These findings challenge the model of a contracting FtsZ ring as pacemaker of cell division. Development of a new technique, SPRAI PAINT, allowed the 3D reconstruction of the spatial organization of the crescentin filament in relation to the cell envelope in living *Caulobacter crescentus* cells (Lew et al., 2011). The approach used EYFP protein fusions for intracellular...
structures and a membrane dye, resulting in a 3D localization precision of 20–40 nm (Figure 5C). In contrast to rod-shaped bacteria, the cell cycle and determination of the division plane is distinct in coccoid- or ovococcal-shaped bacteria. Imaging of the cell wall synthesis sites by a combination of AFM and SIM allowed a first high resolution imaging of septum formation in ovococcal bacteria such as *Streptococcus pneumoniae* (Wheeler et al., 2011).

Further approaches were directed to understand the dynamics of bacterial chromosome and nucleoid-associated proteins (NAP). Lee et al. (2011) used a PALM approach for superresolution microscopy of the abundant NAP HU in *C. crescentus* and observed the redistribution of HU during the cell cycle. Wang et al. (2011) investigated the spatial organization of 5 NAP in *E. coli* using STORM. HU and three further NAP generally showed a scattered distribution over the chromosome, while H-NS was localized in distinct foci (Figure 5D,E). As H-NS acts as silencer of transcriptional activity in bacteria, this observation suggests that H-NS sequesters various regulated regions of the chromosome into clusters. Deletion of H-NS resulted in a gross reorganization of the chromosome.

A further nucleoid positioning system consisting of ParA, ParB and TipN has been recently identified in *C. crescentus* (Ptacin et al., 2010). The group identified a novel bacterial mechanism of the retraction of a polymeric filament that is similar to the segregation of chromosomes in the spindle apparatus in eukaryotic cells. The application of PALM was instrumental to resolve the dynamic action of this chromosome segregation machinery during the cell cycle.

Far less superresolution microscopy data are available for other protein complexes in bacterial cells. The regulation of flagella-mediated bacterial motility depends on a complex regulatory cascade that enables cells to sense chemotactic gradients in a temporal rather than in a spatial manner. Previous work showed that inner membrane chemotaxis sensors, such as the Tar protein, are clustered at the cell poles. Analyses of sensors and interacting cytosolic components of the chemotaxis system by PALM revealed a stochastic, self-assembling mechanism of organization (Greenfield et al., 2009) in *E. coli* cells.

Another new field of application is the analyses bacteria in biofilms. Biofilms are multicellular assemblies of one or more microbial species and the individual cells were embedded in a biofilm matrix that is jointly produced by the cells. The biofilm matrix consists of various combinations of secreted proteins, exopolysaccharides and extracellular DNA. The chemical nature of the biofilm matrix limits ultrastructural analyses approaches. A recent study used STORM to investigate the architecture of *Vibrio cholerae* biofilms (Berk et al., 2012). The relative positions of three proteins involved in biofilm formation and the extracellular polysaccharide were determined in relation of the *V. cholerae* cells with high spatial resolution.

These initial results already demonstrate the considerable potential of superresolution imaging techniques to resolve bacterial microcompartments. Further fruitful applications can be envisaged for unraveling the subcellular organization of bacterial receptor systems and protein secretion systems. Bacteria have evolved a large set of proteins involved in the reception of and response to environmental stimuli. One example is the GGDEF and EAL domain protein involved in synthesis and degradation of the second messenger cyclo-di-GMP, respectively, in response to external stimuli (Hengge, 2009). It will be of future interest to localize the various regulators in a bacterial cell. A further example for prokaryotic complexity
is the multitude of protein secretion systems found especially in Gram-negative bacteria. Pathogenic bacteria use several protein secretion systems to interact with eukaryotic host cells. One example is *Salmonella enterica*, a foodborne gastrointestinal gram-negative pathogen with an invasive and facultative intracellular lifestyle. Functional studies indicate that the adhesion of *S. enterica* to the host cell surface and the subsequent invasion are spatial and temporal controlled processes requiring the action of two distinct bacterial protein secretion systems (Gerlach et al., 2008). A future task will be the resolution of the bacterial secretion systems and target structures on the host cell and the understanding of the dynamics of interaction.

**Conclusions and perspectives**

In addition to near-field excitation, far-field fluorescence imaging beyond the diffraction limit has been made possible by exploiting different mechanisms for switching off fluorophores. Both targeted readout by STED and related techniques as well as stochastic, single molecule localization-based approaches have a huge potential for resolving the spatio-temporal organization of dynamic cellular microcompartments. Key advantages of these techniques are: (i) the spatial resolution <50 nm, and (ii) the compatibility with live cell imaging and the capability to provide information about protein dynamics with nanoscale resolution. STED microscopy is technologically still highly demanding but became affordable since the implementation of continuous wave STED with time-gated detection (s. above). The intuitive imaging approach of STED is conceptually closely related to confocal imaging and no further image analysis is required for obtaining superresolved images. Thus, the application of STED microscopy by cell biologists is straightforward. Moreover, targeted interrogation, as possible by STED, allows focusing on a small region of interest within a specimen, thus providing considerably increased acquisition speed. In contrast, single molecule localization-based imaging is significantly less demanding and the use of different photoswitching and tracking techniques is highly versatile. Its successful application in cell biology, however, requires a deeper understanding of photophysical processes, image acquisition and data analysis. Stochastic fluorophore generation and the limited density of signals, which can be reliably deconvoluted, do not allow interrogating locally with increased speed. The choice of technique thus requires critical assessment of their complementary features as well as the available know-how. Fueled by the success and the increasing demands for the application for unraveling dynamic cellular microcompartments, stimulating innovations in the field can be expected. Increasing the spatial and in particular the temporal resolution will be important. Both STED and single molecule localization-based techniques strongly depend on the photophysical properties of fluorophores such as brightness, photostability and blinking. These currently limit the resolution in time and space and the observation times achieved by these techniques. Therefore, further advances in engineering suitable fluorescent proteins as well as synthetic organic dyes and luminescent nanoparticles and their specific targeting by posttranslational labeling techniques will play a key role, which is highlighted by recent developments in the field (Brakemann et al., 2011; Vaughan et al., 2012; Lukinavicius et al., 2013). With a new generation of highly sensitive sCMOS cameras becoming available, faster image acquisition will become possible, which will increase the time resolution of single molecule pointillism techniques. These will also profit from the development of more powerful image analysis tools (Gould et al., 2012), e.g., algorithms for high-density single molecule deconvolution or compressed sensing (Zhu et al., 2012). Yet, the path towards application of superresolution techniques for addressing biological questions will remain cumbersome, in particular in the field of life cell imaging. So far, only very few cell types and organelles have been subjected to these techniques. Much more experience with suitable biological markers and fluorescence probes for their labeling is required. High overexpression of biological markers and the considerable phototoxicity caused by high-power excitation can substantially bias the biological system. These obstacles need to be overcome by those, who seek a deeper understanding of the cellular organization on the supramolecular level. This will require tight control by complementary techniques. Among these, electron microscopy will play a prominent role, as it provides an independent view of the ultrastructural organization. In contrast, superresolution fluorescence techniques are more suitable for localizing specific proteins within microcompartments. Thus, correlative electron microscopy and superresolution fluorescence imaging (Watanabe et al., 2011; Lidke and Lidke, 2012) may ultimately succeed in providing a meaningful picture of dynamic cellular microcompartments.

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