
Preface

Microscopy imaging experiments have contributed to lay the foundations for modern biological sciences. At the cellular level, the description of numerous cellular components and localization of individual proteins have been achieved through the combination of affinity-based labeling and light microscopy approaches. These techniques are widespread and offer a remarkable versatility for antibody staining but are limited by diffraction in the resolution that they can achieve. In practice, two infinitesimally small light sources cannot be distinguished when they are located closer than ~ 200 nm in the X - Y plane and ~ 500 nm in the Z -direction, limiting the information that can be obtained from biological specimens in conventional light microscopy. Through the use of an electron beam as an energy source, electron microscopy techniques overcame this limitation and have revealed some of the finest details of subcellular structures with accuracy in the Ångstrom range. Although electron microscopy is seen as a gold standard for optical resolution, it comes at the cost of considerably limited possibilities in terms of affinity labeling and live-imaging. As a matter of fact, until recently, neither light nor electron microscopies were able to achieve determination of molecular species at high resolution in living organisms.

The last two decades have witnessed the flourishing development of imaging techniques that contributed to tear apart the diffraction limit of light microscopy in what has been considered a revolution in optical sciences. Altogether these technical advancements, termed “super-resolution imaging techniques,” achieve resolutions beyond the diffraction limit and have proven to combine some of the labeling flexibility of conventional light microscopy with nanometer-scale resolution of electron microscopy, providing new approaches for imaging living physiological samples. As most of the technological breakthroughs that have revolutionized biological sciences, super-resolution imaging approaches have determined a paradigm shift in the way in which experimental approaches are thought, designed, and analyzed, contributing to broaden both practical and theoretical approaches to neurobiological problems.

This book is intended as a comprehensive description of current super-resolution techniques, including the physical principles that allowed their development, some of the most recent neurobiological applications, and selected information for the practical use of these technologies. An historical perspective on light microscopy and general considerations about how the light diffraction limit has been overcome serve as an introduction for the basic principles of super-resolution imaging (Chaps. 1 and 2). Essentially, super-resolution can be achieved (a) with configurations that reveal fluorophores by “depleting” surrounding fluorescent molecules (in stimulated emission depletion, STED; Chaps. 3 and 4); (b) determining the positions of single fluorophores by “making up” the fluorescence signal as in photoactivated light microscopy (PALM) or in stochastic optical reconstruction microscopy

(STORM; Chaps. 5 and 6); and (c) using a structured pattern of illumination for exciting the fluorophores as in structured illumination microscopy (SIM; Chaps. 7 and 8). In addition to these techniques, a number of other exciting developments provide super-resolution images (at least in one dimension): near-field microscopy (Chap. 9), atomic force microscopy (Chap. 10), or super-resolution attempts by X-ray (Chap. 11) and Raman microscopy (Chap. 12). Finally, among super-resolution techniques, electron microscopy photooxidation should be acknowledged as a time-tested but still useful technique to combine fluorescence microscopy with the resolution of the electron microscope (Chap. 13). Higher resolution imaging has likewise raised new challenges for sample preparation and affinity labeling. Two specific chapters cover these issues, highlighting specific technical difficulties and providing useful practical solutions (Chaps. 14 and 15).

Synaptic transmission, which is at the basis of information transfer between nerve cells, is an active topic of research in modern neurosciences. Chemical synapses allow fast and specific neurotransmitter release and are essential for brain functioning. Whereas a number of aspects of synaptic physiology have been investigated by microscopy (e.g., synapse number, localization within neurons, and protein distribution by immunostaining) several features of pre- and postsynaptic contacts cannot be examined at the resolution of conventional microscopy techniques [1].

In the central nervous system, presynaptic terminals are characterized by a complex scaffold of cytomatrix proteins that organize a cluster of presynaptic vesicles filled with neurotransmitter. Upon depolarization, the neurotransmitter content of synaptic vesicles is released in the synaptic cleft and binds the receptors on the postsynaptic cell, mediating information transmission. Intracellularly postsynaptic receptors are held together by a dense proteinaceous meshwork called postsynaptic density. The elaborate synaptic molecular machinery has been studied with nanometer-scale resolution primarily by electron microscopy and immunogold-labeling. Nevertheless, immunostainings in electron microscopy reach a relatively low density and come at the cost of limited resolution of cellular details.

Super-resolution studies have contributed to describe the elaborate molecular architecture of chemical synapses. A STED approach has elucidated that the presynaptic cytomatrix of neuromuscular junctions in *Drosophila* is characterized by doughnut-shaped arrangement of the protein Bruchpilot (BRP, homologue of the presynaptic cytoskeletal structural protein CAST/ERC). In BRP deletion mutants, the T-bar structures that in neuromuscular junctions of *Drosophila* define active zones at the ultrastructural level are lost, calcium dynamics at the synapse are impaired, and neurotransmission is compromised [2]. These results, strengthened by a subsequent study [3], support the idea that BRP is a master regulator of presynaptic active zones by establishing a scaffold that allows T-bar assembly and proper organization of calcium channels.

Multicolor three-dimensional STORM imaging of olfactory bulb and cortex synapses allowed to describe the positioning of a number of pre- and postsynaptic scaffolding proteins with nanometer precision [4]. Among other observations, the authors found that synaptic scaffolding proteins are arranged at a precise reciprocal axial distance from each other with the neurotransmitter receptors localized in-between the pre- and postsynaptic scaffolding proteins. Interestingly, receptors such as NMDA and AMPA have a heterogeneous lateral

distribution among different synapses, suggesting that subpopulations of glutamatergic synapses might cluster receptors to the center or the periphery of the contact depending on functional differences. Additionally, Bassoon and Piccolo, two presynaptic scaffolding proteins, show a defined oriented distribution with the carboxy group and the amino group of proteins, respectively, localized at ~50 and ~75 nm from the synaptic cleft, revealing a previously unknown level of organization of presynaptic active zones.

Along with compelling structural synaptic information, super-resolution microscopy has allowed for the description of key processes in dynamic synaptic function and plasticity. Synaptic vesicles have been largely elusive for conventional microscopy as single organelles due to their ~40–50 nm diameter. A number of super-resolution microscopy approaches allowed describing previously unknown aspects of the vesicle life cycle. A pioneering live-imaging STED study in this field showed that synaptotagmin (an integral vesicular protein) remains clustered at the plasma membrane upon vesicle fusion, indicating that synaptic vesicles maintain multimolecular integrity during recycling [5]. Three-dimensional iso-STED clarified that the very same synaptic vesicles can be used in spontaneous and in activity-triggered neurotransmitter release [6]. Plastic embedding and thin sectioning combined with STED imaging showed that endogenous synaptic vesicle components intermix at very low rates and that fluorescent fusion proteins currently used as reporters of synaptic vesicle localization and activity tend to diffuse and be mislocalized when compared to their native counterparts [7]. Video-rate STED imaging described the movement of single vesicles, revealing that these organelles generally display a random movement, characterized by a lower mobility in the regions that likely correspond to boutons [8]. A subsequent study [9] further investigated this process and revealed that upon endocytosis synaptic vesicles lose their mobility behavior within hours and that neuronal activity block by tetrodotoxin contributes to accelerate vesicle mobility loss. Dual color STED suggested that the process is likely to be tightly regulated and displays an additional layer of control provided by fast endosomal sorting of the vesicles primarily released upon stimulation, the so-called readily releasable pool [10].

Several studies took advantage of super-resolution imaging to describe the dynamic behavior of postsynaptic dendritic spines in living neurons *in vitro* and *in vivo*. STED microscopy of dendritic structures in organotypic hippocampal slices, where YFP was used as a volume marker, besides confirming the well-established paradigm of activity-dependent spine volume increase, revealed details that were previously unknown, such as changes in spine shape and spine head broadening that could suggest physical tightening of pre- and postsynaptic neuronal components functional as well as functional spine maturation [11]. Two-photon STED configurations have applied the same principle to thicker slices [12, 13]. Super-resolution imaging of dendritic spines has been extended to the somatosensory cortex of anesthetized intact living mice where a skull optical window allowed to image living neurons in combination with an upright STED setup [14]. Both PALM and STED approaches allowed following the dynamic reorganization of actin molecules within single dendritic spines, revealing that in dendritic spines, actin molecules are organized in subdomains and form bundles in the neck of spines, undergoing activity-dependent changes [15–18].

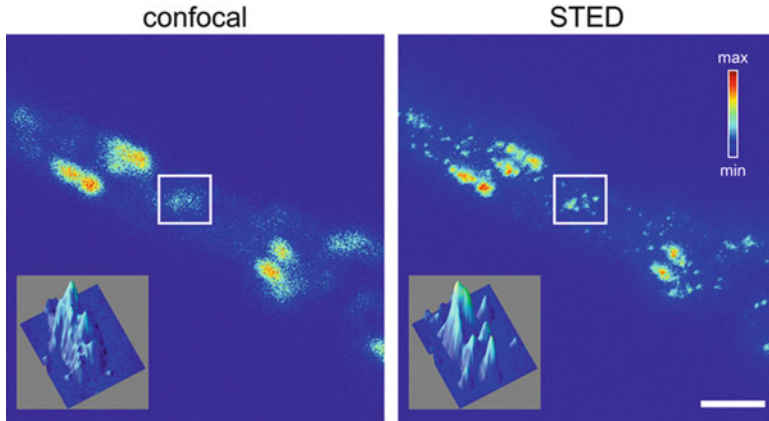


Fig. 1 Comparison of confocal and STED imaging. Primary hippocampal neurons were labeled for the synaptic vesicle marker Synapsin I and imaged with a commercially available Leica TCS STED system in confocal mode (*left*) and in STED mode (*right*). Insets: three-dimensional surface rendering of the boxed regions (820×760 nm). Note that in the confocal image only large spots are detected while STED imaging allows resolving smaller structures within otherwise unresolved regions. Scale bar 1 μ m

We believe that the “super-resolution revolution” will not just increase the resolution in the imaging capabilities of neuroscientists (Fig. 1), but it will also contribute to change the perspective in which cellular biology processes are understood at the nanometer scale.

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