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Advances in Light-based Imaging of Three-Dimensional Cellular Ultrastructure

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Abstract

Visualization methods are key to gaining insights into cellular structure and function. Since diffraction has long confined optical microscopes to a resolution no better than hundreds of nanometers, the observation of ultrastructural features has traditionally been the domain of electron microscopes (EM). In the past decade, however, advances in super-resolution fluorescence microscopy have considerably expanded the capability of light-based imaging techniques. Advantages of fluorescent labeling such as high sensitivity, specificity, and multichannel capability, can now be exploited to dissect ultrastructural features of cells. With recent methods capable of imaging specific proteins with a resolution on the order of a few tens of nanometers in 3-dimensions, this has made it possible to elucidate the molecular organization of many complex cellular structures.

Introduction

Many structural components of cells have nanometer-scale dimension: lipid bilayers are ~5 nm in thickness; a typical globular protein domain measures a few to several nm in size; eukaryotic cytoskeletal filaments range from 8 nm (actin) to 25 nm (microtubules) in diameter. Thus, the nanoscale (~10–100 nm) is the relevant length scale for understanding their assembly into higher-order cellular machinery. Although electron microscopy (EM) has long been the primary tool for observing nanoscale structures, several limitations remain difficult to overcome, such as limited molecule-specific labeling and difficulties with live or hydrated specimens. Therefore, there is a need for alternate imaging methods that address these limitations while retaining high spatial resolution comparable to EM.

Since diffraction limits the smallest focal volume into which light can be focused, i.e. the point spread function (PSF), to ~250 nm in the lateral image plane and ~500 nm along the optical axis, spatial details smaller than the PSF dimension cannot be resolved by conventional light microscopes (CLM). Super-resolution microscopy (SRM) denotes a group of light-based optical imaging techniques that aim to achieve higher spatial resolution than the diffraction-limited performance of CLM. These methods thus promise to combine the strengths of CLM—non-invasiveness, compatibility with physiological conditions, and

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highly sensitive and molecule-specific fluorescent labeling—with EM-like high resolution performance.

The principles of operations of major SRM approaches have been covered in a number of recent reviews[1–4]. Briefly, these methods (Fig. 1) surpass the diffraction barrier via: i) the reduction of the effective PSF size by stimulated emission depletion (STED)[5–7]; ii) the extraction of high spatial frequency through the use of periodic excitation patterns (Structured Illumination Microscopy: SIM) [8]; and iii) the precise localization[9] of individual labeled molecules (Localization Microscopies, such as F/PALM: Fluorescence/Photo-Activated Localization Microscopy[10,11], Stochastic Optical Reconstruction Microscopy: STORM[12], Ground-State Depletion and Individual Molecule Return Microscopy: GSDIM[13]). Within the past few years, there has been a great deal of interest in these techniques, with several SRM systems being commercialized by major microscope manufacturers.

Since most cellular structures are inherently 3-dimensional, SR capability in 3-D is critically important for biological applications. However, until recently SRM typically achieved super-resolution in 2-dimensions only. Indeed, since the axial resolution of most microscopes is far worse than the lateral resolution, 3-D resolution enhancement represents an ongoing challenge in optics. In this article we review advances in SRM over the past few years, with particular attention to the extension of SRM performance into the 3-D realm, recent progress in fluorophore technologies, and emerging applications of SRM to dissect cellular ultrastructure.

Recent Developments in Three-Dimensional Super-Resolution Imaging

A number of 3-D SRM approaches are extensions of 2-D SRM concepts and can be implemented on common inverted microscope platforms. For SIM, a diffraction grating has been used to generate an excitation pattern in 3-dimensions[14,15] (Fig. 2a, bottom). Such patterned excitation was applied at different angles and different axial depths so that the resulting raw image sets contained double the spatial frequency information in all 3 dimensions. Fourier-transform-based processing then allowed reconstruction of a super-resolved image with 2 times resolution improvement beyond the diffraction limit. For Localization Microscopies which are based on single PSF analysis, several approaches have been implemented to either measure the axial profile of the PSF or modify the PSF so that axial information is encoded in the PSF. In the biplane FPALM method[16], a beamsplitter separated the image of single molecules into two channels, focused at slightly different axial planes ~350 nm apart. The axial position could then be determined from the single molecule peak widths in the two detection planes. In the astigmatic approach[17,18], a weak cylindrical lens was used to mildly defocus the image beam along one direction, causing the PSF to vary in ellipticity as a function of the axial position. In a related method, the PSF was modified by a phase-sensitive spatial light modulator into a pair of spots that rotate around the z-axis, i.e. a double-helix[19]. For these methods, the PSF ellipticity or the double helical angular orientation for each molecule, respectively, were then used as z-coordinate read-outs. Altogether, while these techniques allow 3-D SRM on relatively simplified optical setups, their axial resolutions are still typically a few times worse than their lateral resolutions, due to the inherent limitations of the single objective lens design. Greater axial resolution has been attained using a ‘virtual volume’ method[20] whereby the sample is deposited on a specialized micro-grooved mirror so that the virtual image (side-view reflection) is observed side-by-side with the real image. This approach provides more isotropic resolution, although a much smaller field of view can be imaged.

Through the use of dual opposed objectives lens (4-pi) configurations[21, 22], significant axial resolution gains have been demonstrated for STED[23], SIM [24], and Localization Microscopies [25,26]. Although these require more complicated instrumentation and are accompanied by a sample thickness limitation (clear optical access is needed from both sides of the sample), substantial resolution gain several times that of related single lens based approaches can be achieved. For example, I⁵S microscopy combines grating-generated structured illumination with a 4-pi setup to achieve sub-100 nm resolution in 3-dimensions[24]. In another example (Fig. 2b), isoSTED[23] confines the effective focal volume in all 3 dimensions, resulting in a nearly isometric ~40 nm PSF, providing much enhanced resolution. When adapted to Localization Microscopy, the 4-pi configuration enables interferometric measurement of single molecule axial position such as in Interferometric Photo-Activated Localization Microscopy (iPALM)[25] or 4Pi-Single Marker Switching Microscopy (4Pi-SMS)[26]. In this scheme, fluorescence emission from each molecule is collected through both objectives and recombined using a specialized beamsplitter to produce an interference signal. Since the axial position of the emitter is directly proportional to the pathlength differences through the objective lenses to the beamsplitter, the phase of the interference signal contains axial information which can be measured by simultaneous multiphase projection [25] (Fig. 2c). The inherent sensitivity of interferometry allows axial resolution almost two times higher than the lateral, achieving better than 20 nm resolution [25,26].

Another active frontier in 3D SRM is in extending the imaging depth. This is particularly important since many physiological processes occur in complex 3-dimensional matrices or tissue milieu, but the axial range of most current SRM methods are limited to ~1 μ m or less[16,19,25–27]. Since non-ideal optical properties of thick biological materials pose tremendous difficulties for optical imaging, the main challenge lies in combining an SRM method with an optical sectioning scheme. For STED, the inherent confocal-based optical sectioning ability can be enhanced by 2-photon excitation[28,29], as well as by adaptive optics correction for sample-induced aberration[30]. By using a grating and a coupling lens with a femtosecond laser, either 2-photon activation[31] or excitation[32] can be confined to a specific narrow axial plane via the temporal focusing effect, allowing Localization Microscopy imaging several microns deep into samples[31]. Furthermore, via rapid scanning of the 2-photon beams[32], 3-D PALM imaging can be carried out more than 8 μ m into the sample. In addition, SIM has proved to be particularly useful in combination with Bessel beam[33] selective plane illumination microscopy (SPIM)[34], which excites the sample from the side with thin (<500 nm) light sheet to reduce photobleaching and background. SPIM may also be compatible with future Localization Microscopy implementations.

Super-resolution Fluorophores

Even though the optical aspects of SRM tend to garner more attention in the field, in practice, the resolution limits of each approach is very much dependent on the properties of available fluorophores. Thus, research into fluorophore improvement and/or optimization of their photophysical properties bears significant importance for SRM advances. This is most critical for Localization Microscopies because the brighter the fluorophore, the more precise each molecule can be localized[9]. At the same time, suitable fluorophores must also exhibit photoswitching behaviors to enable high density labeling[10–12]. Although initial SRM demonstrations and applications were limited to a few photoactivatable fluorescent proteins (PA-FPs)[35–38] or carbocyanine dye pairs such as Cy3-Cy5[39,40], the palette of suitable PAFPs and dyes has since greatly expanded. For example, the PA-FP PAmCherry1 has been developed, which when used in conjunction with PA-GFP, allows simultaneous dual color photoactivation imaging in living cells[41], while mEos2 has been developed to optimize

brightness while minimizing oligomerization artifacts[42]. Furthermore, photoswitching of synthetic fluorophores has been better characterized[43], and has been found to be more common[44] than initially supposed. Cyanine dyes such as Cy5, Alexa647[45], or ATTO655[46] are capable of photoswitching via thiol reactions under reducing conditions[47]. Noteworthy, several commercial dyes photoswitch in aqueous buffer[44] under ambient oxygen or mildly reducing conditions[46]. Thus, sample preparation can be greatly simplified. In general though, the brightness of PA-FPs or synthetic fluorophores used in the photoswitching mode is still inferior to the that of conventional non-switching fluorophores used in single molecule biophysics experiments, where they can achieve a few to sub-nm localization accuracy[48,49], suggesting that there is significant headroom for further improvement in resolution in Localization Microscopy techniques by improvements to photoswitchable fluorophores.

The applicability of other SRM modes such as STED and SIM also benefit significantly from improvement in fluorophore properties. Early applications of STED were performed using photostable dyes such as Atto532[5,50] which tolerate the high level of energy needed to deplete the excited state. Recently, however, further improvements in spectral range and laser source optimization have expanded the list of suitable dyes, enabling STED imaging of the green fluorescent proteins in live *Caenorhabditis elegans* [51]. Meanwhile, the resolution of SIM can also be greatly enhanced if nonlinear saturable fluorophores [52] are used.

As SRM techniques reach into the nanoscale regime, the actual dimension of the label itself becomes non-negligible. Although synthetic fluorophores are commonly introduced via antibodies, this convenience is traded off against resolution. Since an antibody molecule is >10 nm long, using both primary and secondary antibody can be expected to add a few tens of nm or more in localization uncertainty. In terms of size, genetically encoded PA-FPs are advantageous since their diameters are at most 5 nm. However, PA-FP brightness is still inferior to synthetic fluorophores, prompting development of alternative fusion tags that utilize small globular protein domains with specific small molecule binding sites to allow delivery of adduct-dyes with superior photophysics. Recent examples applied to live cell SRM include the SnapTag®[53–56] and trimethoprim-DHFR (dihydrofolate reductase) systems[57]. By using exogenous far-red dyes, this approach, either by itself or in combination with PA-FPs, should facilitate live cell triple- or quad-channel SRM.

Light-based Visualization of Cellular Ultrastructure

The sensitivity and specificity afforded by fluorescent labeling makes SRM particularly useful in spatially and compositionally resolving complex biological structures. Recent applications of SRM, discussed below, have been particularly informative in delineating cellular architecture at the nanoscale to complement what is known and not known from EM studies. However, it is important to emphasize a major difference in the information content provided by SRM techniques and the more familiar EM. Unlike in EM where rich ultrastructural context can be gleaned, since membranes and numerous structures are visible, SRM, with its highly specific fluorescent labeling, primarily visualizes the positions of molecules that are labeled. Although many SRM studies have thus far focused on technical demonstrations and generally made use of only a few labeled protein species, for cell biological applications, researchers should probe for as many species as practicable, since the salient features of the structures of interest may not be discernible with only one probe. Furthermore, biological applications of SRM require a clear marker to identify the structure of interest for analysis. While this can be relatively straightforward for well-defined organelles such as the focal adhesions [58] or neuronal synapses[59] for which the structures are synonymous with a high density of the protein of interests, for membrane-based

organelles or structures with low labeling density, it may be difficult to depend only on SRM for spatial clues. This has spurred the development of correlative SRM-EM[60] where the high-density molecule-specific SRM data can be directly overlaid with context-rich EM to help identify ultrastructural features of interest.

One very important recent application for SRM has been in prokaryotic cell biology. Since the internal structures of prokaryotes are much more challenging to visualize by CLM due to their small cell sizes, typically only a few microns in length, SRM provides an immediate remedy to this limitation and has been actively employed by several research groups. In one example, Localization Microscopy of FtsZ, the cytoskeletal element involved in cell division in *Escherichia coli*, revealed for the first time that this protein forms a compressed helix at the division plane, suggesting that this “Z-ring” is composed of a loose bundle of FtsZ protofilaments [61]. In another study, PALM was used in conjunction with modeling to map the cellular locations of three proteins central to *E. coli* chemotaxis, indicating that chemotactic receptors self-organize into signaling clusters[62]. Localization Microscopy was also used to determine the cell-cycle dependent changes in organization of HU, a nucleoid-associated protein (NAP) critical to chromosomal architecture in *Caulobacter crescentus* [63]. This showed that HU becomes highly clustered in predivision cells, suggesting the exciting possibility that chromosome condensation may also occur in prokaryotic cells, as it is well known to do in eukaryotes. Altogether, experimental access provided by SRM help to contribute to newfound recognition that prokaryotic cells possess dynamic and intricately organized ultrastructure, in contrast to the old notion that these “simple” cells are mere packets of diffusing biomolecules.

Several SRM applications to eukaryotic cell biology have also been reported recently. For example, 3D-STORM has been used to image the clathrin-dependent endocytic machinery generated in a cell-free system, revealing the roles and organization of specific F-BAR proteins in endocytosis and membrane invagination [64]. In another example, highlighted in figure 3, the molecular architecture of integrin-based focal adhesions (FA) [58] was dissected by iPALM[25]. Through their function as dynamic, adhesive organelles that mediate adhesion and force transmission between the actin cytoskeleton and the extracellular matrix, FAs play important roles in cell migration, mechanosensing, and many signaling pathways including those that regulate cell fate and differentiation [65]. However, despite early recognition of their physiological importance, the underlying ultrastructure of FAs had proven elusive. Because FAs contain a variable ensemble of more than a few hundred protein components [66] all confined within highly dense and compact plaques measuring <200 nm along the smallest axial dimension [67, 68], efficient probing by immuno-metal EM methods are hindered. Since the probe density and specificity limitations are largely circumvented by fluorescent protein technologies, with iPALM it was possible to reveal for the first time that FA proteins are distinctly organized along the axial dimension (Fig. 3a) [58]. The actin cytoskeleton was observed to be separated from the integrin cytoplasmic domains by a gap of ~30–40 nm, within which contained strata of FA proteins such as focal adhesion kinase (FAK), paxillin, vinculin, and zyxin (Fig. 3a). Furthermore, the placement of PA-FP tags at either the N- or C- terminus of a large FA adapter protein talin allowed determination of its orientation, revealing a highly polarized organization, suggestive of the role of talin as a direct integrin-actin linker that could also help organize the FA multilayer architecture (Fig. 3d–g). Interestingly, the interposition of talin and vinculin between actin and integrin (Fig. 3a) has a parallel in other studies in which these proteins exhibit interdependent dynamics [69] and localization [70] as well as interdependence in control of adhesion function [71]. Taken together, these results corroborate the notion that FAs serve as spatiotemporally regulated molecular clutches during cell migration [58,69]. The FA example above also highlight the benefits of using

probes with known or well characterized tag locations, which enable determination of molecular orientation within the context of a cell.

In another recent SRM application to neurobiology, the molecular organization within the neuronal synapses were probed by 3D-STORM[59]. By immunolabeling the N- and C-terminal domains of the large scaffold proteins Piccolo and Bassoon, SRM imaging revealed their highly directional orientations, reminiscent of talin in FAs, suggesting that the two structures may share a common architectural motif despite different identities of the proteins involved. It remains to be seen whether other dense protein structures at membrane-cytoskeleton interfaces such as the adherens junction or the immunological synapse share similar architecture. Furthermore, these studies suggest that in addition to localizing –N and –C termini of target proteins, SRM analysis should be applicable to other types of fusion constructs containing, for example, domain deletions, rearranged domains, or site-specific mutations.

Concluding Remarks

As cellular structures rarely consist of single or a few biomolecular species, SRM imaging should be a particularly useful tool for probing the structure-dynamics-functions relationship at the supramolecular level. There is a vast range of biological systems, otherwise laborious to probe by EM, where SRM techniques have the potential to contribute to better understanding of their structures and functions. Enhancement of SRM with multichannel capabilities will be particularly useful to localize multiple protein species relative to one another to establish the nanoscale blueprint, i.e. the generalized molecular archetype that captures the basic organization of components. Importantly, examples of SRM applications described herein suggest that many more cellular structures should now be within the capabilities of current technologies. Such knowledge of the molecular architecture will also be essential for further application of SRM to live cells, especially since novel dynamics that will be revealed by SRM are expected to occur on the nanoscale.

Beyond the static model, further insights into biological functions depend on the ability to observe and perturb the assembly, transformation, and disassembly dynamics of cellular ultrastructures. These entail not only raw imaging capability but also the ability to detect and analyze dynamic, structural, and compositional heterogeneity within each systems. Here, the challenges beyond pushing the speed, resolution, and ease-of-use of the imaging techniques lie in the inevitable fact that higher resolution data means an exponential increase in data size. Even for SIM and STED, which output conventional image datatypes, the increase in data volume is still large (i.e. 2x gain in resolution in 3 dimensions necessitates 8x increase in data size for the same coverage of a cell). This challenge becomes much greater for Localization Microscopy data. As an example, a typical single frame of 2D-localization microscopy data can contain hundreds of thousands of individual molecular coordinates at minimum. For 3-D live imaging, the data volume will become correspondingly more massive. Hence, we anticipate more research activity in the development of computational and analytical tools for the mining of these large datasets.

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2-dimensional super-resolution **Figure 1. Principles of 2-Dimensional super-resolution microscopy**

a) Structured illumination (SIM) relies on image analysis in the spatial frequency domain. High resolution information (i.e. high spatial frequency) in the image (unknown, left) is obscured by diffraction. By using a known periodic excitation pattern (center), the frequency difference between the known pattern and the unknown image features give rise to lower frequency beat effects (Moiré' fringes) which can be observed. A super-resolved image is reconstructed from a Fourier-Transformed based analysis of raw data sets, taken at multiple angular orientations of the illumination patterns. **b) Stimulated Emission Depletion (STED)** reduces the effective size of the point spread function (PSF). Fluorophores are excited by a diffraction-limited excitation beam (normal PSF). By using a donut-shaped depletion laser beam at a slightly longer wavelength, excited fluorophores in the outlying ring rapidly de-excite by stimulated emission, leaving only the fluorophores near the PSF center to emit. Measured fluorescence signal originates from a much smaller volume, and thus STED can be readily employed with point-scanning confocal microscopy. **c) Localization Microscopy (F/PALM,STORM)** depends on photoswitching properties of the fluorophores. Although multiple fluorophores that situate close together cannot be resolved if they emit at the same time (diffraction-limited, green), random photoswitching can be exploited so that at a given instance, molecules are singly or sparsely switched on and can be detected in isolation. Data acquisition involves recording typically several thousands of frames of single molecule movies. Individual fluorophores appear as a diffraction-limited peak in the raw image (**d**). Each peak is computationally detected and its lateral (xy) coordinates determined by fitting to a PSF model function (e.g. a 2-dimensional Gaussian). In general, the localization precision is inversely proportional to the fluorophore brightness [9], and the uncertainty in the coordinate is typically represented by the peak width in the rendered image (**e**). Thus, a super-resolved image (**c**, inset) can be reconstructed by plotting together the aggregated list of the high-precision localized coordinates. (SIM schematics reproduced from [52], with permission from National Academy of Sciences, USA)

3-dimensional super-resolution

Figure 2. Optical configurations and principles of 3-D super-resolution microscopy

a) 3-D SIM employs a diffraction grating and a polarizer in the epi-fluorescence excitation pathway (**a**, top) to create periodic patterns both in the lateral plane and along the axial dimension (**a**, bottom, xz cross-section of the 3-D illumination pattern). A super-resolved image is computationally restored by Fourier analysis from a raw image set taken at different angles and different axial positions. **b) isoSTED** uses dual opposed objective lens (4-pi configuration, **b**, top). The excitation beam (green, **b**, top) and co-aligned depletion beams illuminate the sample from both sides. Two depletion beams (not shown) are used to reduce the effective PSF radius along both lateral (xy) and axial (z) dimensions, resulting in nearly spherical PSF spot (**b**, bottom). **c) iPALM** relies on a 4-pi configuration and simultaneous multiphase interferometry via a customized beamsplitter (**c**, top). The emission from each single molecule propagates through both the top and bottom objectives. The difference in the optical pathlengths between the two beams is directly proportional to the axial position of the molecule. The two beams are recombined by a 3-way beamsplitter which yield 3 projected beams with a mutual phase difference of $\sim 120^\circ$. The phase of the recombined signal thus encodes the axial coordinate, which is determined from the intensity ratio of the same molecule between the 3 cameras (**c**, bottom), whereas single molecule localization is used for lateral coordinates. (Obj: Objective lens, CCD: Charge-Coupled Device Camera, BS: Beamsplitter, GM: Galvo mirror, DM: Dichroic Mirror; 3D-SIM schematics reproduced from [14], with permission from Elsevier Press; isoSTED figure from [23], Nature Publishing Group; iPALM figure from [25], National Academy of Sciences, USA)

Figure 3. Ultrastructural analysis of integrin-based focal adhesions by 3-D super-resolution microscopy

a) Multilaminar organization of focal adhesions. Axial positions (Z_{center}) of various proteins in focal adhesion is determined from the peak of the axial position histogram, and displayed as notched box and whisker plots. The distances are measured from the coverglass substrate.

b) Schematic model of the focal adhesions molecular architecture. A model is drawn based on the protein position measurement by iPALM. The adapter protein talin is portrayed as forming an array of directional tethers spanning the gap between integrin and actin, based on the imaging of the N- and C- terminal tdEos fusion tags of talin (c). (d-f) iPALM image reconstructions of localizations of the probes shown in (c), scale bar = xx microns, color encodes distance from the coverglass surface in nm. (d, e) The talin-N probe reports N-terminal position as being localized close to the cytoplasmic tails of integrin, whereas the talin-C probe (f, g) reports position up to 40 nm higher (f-g). Sideview projections of FAs denoted by white boxes in (d, f), are shown in (e, g). Both probes are well localized with little extent of overlap, suggesting that talin is highly oriented within focal adhesions. (Figures reproduced with permission from [58], Nature Publishing Group)