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Subdiffractive microscopy: techniques, applications, and challenges

Brian R Long^{*}, Danielle C Robinson, and Haining Zhong

Vollum Institute, Oregon Health & Science University, Portland, Oregon 97239, USA

Abstract

Cellular processes rely on the precise orchestration of signaling and effector molecules in space and time, yet it remains challenging to gain a comprehensive picture of the molecular organization underlying most basic biological functions. This organization often takes place at length scales below the resolving power of conventional microscopy. In recent years, several “superresolution” fluorescence microscopic techniques have emerged that can surpass the diffraction limit of conventional microscopy by a factor of two to twenty. These methods have been used to reveal previously unknown organization of macromolecular complexes and cytoskeletal structures. The resulting high-resolution view of molecular organization and dynamics is already changing our understanding of cellular processes at the systems level. However, current subdiffractive microscopic techniques are not without limitations; challenges remain to be overcome before these techniques achieve their full potential. Here, we introduce three primary types of subdiffractive microscopic techniques, consider their current limitations and challenges, and discuss recent biological applications.

The integration of information within a cell relies on the specific and dynamic organization of individual proteins and macromolecular complexes. Over many decades, data on molecular organization and interactions has been collected through indirect measurements, such as biochemical assays. However, in order to understand cellular processes at the systems level, there is a growing need to directly measure the precise spatiotemporal organization of proteins within the cell.

Fluorescence light microscopy is widely used to study cellular and subcellular organization because of three critical advantages: 1) It provides high contrast information on appropriately-labeled targets over several orders of magnitude in length and time. 2) It produces quantitative results under the appropriate conditions. 3) The development of functional sensors has allowed for live monitoring of specific signaling events. Despite these advantages, conventional fluorescence microscopy falls short when the relevant length scale approaches the size of individual macromolecular complexes (~10–200 nm). A fundamental limit called the diffraction limit prevents the resolution of details below ca. 200 nm (see **Background**). Fortunately, recent years have witnessed a revolution in fluorescence microscopy with the development of several subdiffractive fluorescence imaging modalities^{1–4}. These “superresolution” microscopy techniques utilize novel illumination schemes, and/or data collection and analysis principles, to extend the resolution two- to twenty-fold beyond the diffraction limit in biological samples.

As the superresolution microscopic technologies mature, they are becoming available to biologists for investigating cellular and subcellular organizations at a new level of detail.

Correspondence should be addressed to BRL (brianl@alleninstitute.org) or HZ (zhong@ohsu.edu).
^{*}current address: Allen Institute for Brain Science, Seattle, Washington, USA.

However, it is important to note that these methods are not without limitations. For example, the choice of fluorophore must be carefully considered and the selection is often limited. In addition, with improved resolution comes the need for high quality sample preparation to adequately label and preserve native macromolecular structures. Here, we introduce three major classes of subdiffraction imaging techniques: stimulated emission depletion microscopy (STED)^{5,6}; structured illumination microscopy (SIM)⁷; and localization-based methods (LBM)^{8–10} such as photoactivated localization microscopy (PALM), fluorescence PALM (fPALM), and stochastic optical reconstruction microscopy (STORM). As many excellent reviews already cover the technical details of these methods^{1–4,11–16}, we focus on their respective advantages and disadvantages, as well as the challenges for each method and the overall field. We also discuss the rapidly growing role of subdiffraction microscopy across a wide range of biological research, emphasizing biological results over technical achievements.

Background: Diffraction limit in biological imaging

The diffraction limit is a fundamental resolution limit of conventional light microscopy first described by Abbe in late 1800s. Due to the wave properties of light, a point source of light in the sample plane of the microscope cannot be re-focused to a single point in an image. Instead, the light is diffracted to form an intensity distribution called the point-spread function (PSF), whose size is proportional to the wavelength λ and inversely proportional to the numerical aperture (NA) of the objective. In the late 1800s, Abbe studied the effect of diffraction on image formation and described the Abbe limit, $r_{\min} = 0.5\lambda/\text{NA}$, which reflects the smallest features size that can be reproduced in an image. For a typical high-NA lens (e.g., NA = 1.4) and the emission wavelength of GFP (509 nm), $r_{\min}$ is approximately 180 nm. For wide-field fluorescence microscopy, the effect of the diffraction limit is conceptually equivalent to adding a blur across the entire image. As a result, if two small objects are located within the width of a PSF, their PSFs will sum up and they cannot be distinguishable from each other (Fig. 1A). In confocal microscopy, the image resolution is largely determined by the size of the focused excitation light spot, which is also characterized by the PSF. The smaller the excitation PSF, the more precisely the image reports the origin of a fluorescence signal. Two objects can only be resolved by confocal microscopy if they are separated by at least 180–300 nm. For in-depth discussions on the diffraction limit, and the related Rayleigh and Sparrow criteria, we refer the reader to the references 13 and 17.

Many biological systems within the cell are organized at the scale of tens of nanometers, which is beyond the diffraction limit. Electron microscopy (EM) and biochemical protein-protein interaction analyses have provided researchers an intriguing glimpse of molecular interactions and organization at the nanoscale. For example, both EM and biochemical assays suggest that neuronal synaptic proteins are organized into macromolecular complexes in which the precise composition and localization are thought to determine synaptic function (see Fig. 1B for an EM image of a synapse)^{18,19}. However, when it comes to deciphering the precise protein organization within synapses and other biological systems, EM methods encounter difficulties in reporting protein identity, while biochemical methods lack spatial information. Furthermore, these techniques are incompatible with live imaging.

Subdiffraction Imaging: methods, practical capabilities, and limitations

In response to the limitations of conventional fluorescence microscopy, EM, and biochemical techniques, several fluorescence microscopic methods have emerged to reach beyond the diffraction limit. The three methods described below are now commercially

available and have already begun to enhance our understanding of complex biological systems (Fig. 1C).

SIM

SIM utilizes patterned illumination and computational reconstruction to achieve resolution up to two times better than the diffraction limit^{7,17} (Fig. 1D). In SIM, the sample is illuminated with grating-patterned excitation light. This patterned excitation combines with the sample's fluorescence distribution to produce a Moiré pattern (Fig. 2) that contains the spatial "beat frequency" between the sample and the patterned excitation light. High spatial frequency information of the sample that is lost in conventional microscopy can be detected by imaging the Moiré patterns, which have a lower spatial frequency than the sample. The structured excitation pattern is shifted to 3–5 positions and rotated to 3–5 angles relative to the sample, with an image collected at each grid pattern position and angle. Subdiffractive information is then computationally extracted to generate a final image with up to two-fold enhanced spatial resolution. Existing literature includes detailed reviews of SIM^{17,20} and clear explanations of the principles behind the technique^{7,21,22}. To date, 3D-SIM (with a two-fold resolution improvement in the z-dimension) has been developed, as have multi-color SIM and live cell SIM^{22–24}. Microscopes with 3D and multicolor SIM capabilities are commercially available (e.g., from Zeiss, Nikon, and Applied Precision).

Resolution limits: fundamental and practical—Conventional SIM is limited to a two-fold improvement beyond the diffraction limit because the excitation grating pattern is itself diffraction limited. To overcome this limitation, nonlinear SIM (NL-SIM) uses patterned excitation at saturating illumination intensity to allow the Moiré pattern images to contain higher spatial frequency information^{21,25}. A four-fold resolution increase over the diffraction limit has been demonstrated for biological samples²⁵. Although NL-SIM techniques could theoretically allow unlimited resolution, phototoxicity and sample bleaching under the saturating light intensity are practical limitations, and NL-SIM has not yet been commercialized.

Practical Considerations—A major advantage of SIM is the simplicity of sample preparation and its ease of use. Fluorophores and samples that are suitable for confocal microscopy typically also work well for SIM. On a Zeiss Elyra system, image collection requires typically 5 positions at 3 grating angles per z position and ~50 ms exposures, resulting in acquisition times of approximately 60s for a 10 μm z stack with images collected every 125 nm in the z dimension (Stefanie Kaech-Petri, personal communication). SIM images are generated after collection, with processing time comparable to image collection time. SIM uses wide-field detection that can image relatively large fields of view. However, SIM may encounter difficulties in thick, light scattering tissues because wide-field detection does not reject out-of-focus or scattered fluorescence. The need for multiple exposures to reconstruct a SIM image makes bleaching and phototoxicity potential concerns especially in live imaging. A recent technique combining SIM with Bessel beam planar illumination has ameliorated many of these problems, but it requires complicated custom instrumentation²⁶. In addition, recent advances that implement multifocal illumination and optical processing can achieve a 100 Hz frame rate without the need for digital processing^{27, 28}.

STED

STED microscopy is a scanning imaging modality similar to confocal microscopy^{5,6,29}. In STED, however, the effective size of the excitation spot is reduced using a well-aligned second laser that forms a donut shaped light spot to quench fluorescence emission around the center of the excitation PSF (Fig. 1D & Fig. 2). The quenching is achieved via

stimulated emission (the same mechanism responsible for laser generation), which forces fluorescence emission only at the wavelength of the second laser. The stimulated emission, typically at a longer wavelength outside of the fluorescence detection band, will not be detected. As a result, detectable fluorescence emission only originates from center of the donut, thereby creating a smaller effective excitation PSF. The excitation beam and depletion doughnut are scanned in a raster pattern throughout the sample to create a subdiffraction image without any need for post-processing. Multi-color STED, live and 3D STED imaging have also been demonstrated^{6,30–32}. An important development is the employment of two-photon excitation, which allows the use of STED deeper in tissue while reducing photobleaching^{33–36}. In addition, the use of continuous-wave lasers may significantly reduce cost and complexity associated with the mode-locked, pulsed laser originally used with STED⁶, and gated detection substantially reduces the power needed for imaging³⁷. Currently, the 2D version of STED (Fig. 1D) is commercially available through Leica.

REversible Saturable Optical Fluorescence Transitions (RESOLFT) is a related technique that uses reversibly photoswitchable fluorophores, which can be switched on and off using particular wavelengths of light^{12,14}. The sample is first switched on with a diffraction-limited activation light spot, followed by a donut-shaped laser spot to switch off fluorescence to reduce the size of the activated spot, and then imaged with diffraction-limited excitation light. As a result, the fluorescence emission only originates from a spot much smaller than the size of the excitation PSF. Recent improvements in reversibly-photoswitchable fluorescent proteins have led to demonstration of 50 nm structures in live cells using RESOLFT³⁸. Readers interested in the principles and practice of STED and related techniques are directed to several reviews on the subject^{2,29,39}.

Resolution limits: fundamental and practical—The PSF size of STED is given by

The PSF size of STED is given by $\sigma_{\text{STED}} \approx \frac{\lambda}{2 \text{NA} \sqrt{1+I/I_{\text{sat}}}}$, where I is the light intensity of the depleting (donut) light spot and I_{sat} is the intensity required for 50% of the fluorophores to be depleted. The resolution is therefore mainly determined by the applicable laser power, which is limited by practical factors such as bleaching and phototoxicity. For biological samples, STED resolution of more than 50 nm is typically reported, although images with features down to 20 nm have been demonstrated in fixed samples using a modified form of STED⁴⁰. STED has also been applied to *in vivo* imaging, with one study demonstrating ~70 nm lateral resolution 10–15 μm deep in the cortex of a living mouse⁴¹.

Practical Considerations—Fluorophores used in STED and RESOLFT must undergo many cycles of excitation/activation and depletion without productive fluorescence emission. Specialized fluorophores that are very stable under these demanding imaging conditions (e.g., Dylight and Atto dyes⁴², and rsEGFP^{38,43} have been optimized for STED and RESOLFT imaging, respectively), although conventional fluorophores, such as Alexa dyes, GFP, and YFP have also been used^{31,32,44}. In addition, the high power density required for STED fluorescence depletion (5–200 MW/cm^2 for continuous-wave STED)^{38,42} may produce photodamage and phototoxicity especially during live imaging. This problem is alleviated in RESOLFT because rsEGFP can be turned on and off at relatively low light intensity, although the choice of fluorophores (e.g., for multi-color imaging) is limited.

STED data acquisition is relatively fast for small fields of view. However, the imaging speed decreases rapidly as the field of view increases. This is because the required number of pixels and the imaging time will increase proportional to the area of the field of view (See

Focus Box). RESOLFT imaging speed is further limited by the rates of fluorophore switching – the fluorophore switching cycle must be completed at each pixel before continuing the raster scan. To enhance the imaging speed, a recent study created a large array of over 100,000 spots for RESOLFT imaging, reducing the required scan range and allowing detection with a sCMOS camera⁴⁵, albeit at the expense of increased imaging laser power to generate the multiple RESOLFT spots and higher sensitivity to light scattering.

FOCUS BOX

Sampling and resolution in subdiffraction imaging

The Nyquist-Shannon sampling theorem states that the sampling frequency of any signal must be more than twice as fine as the desired resolution. This criterion presents profound implications for subdiffraction imaging. There are two sampling steps involved in biological imaging: the labeling of the sample and the imaging of the labels. Both of these steps have to meet the Nyquist criterion for successful subdiffraction imaging.

For the labeling step, the density of fluorescent labels must be high for high-resolution imaging. Specifically, the distance between individual labels must be less than half of the desired resolution. Thus, while a labeling density of $100/\mu\text{m}^2$ (100 nm averaged inter-label distance) is sufficient for a lateral resolution of diffraction limited imaging (~200 nm resolution), $10,000/\mu\text{m}^2$ would be required for a resolution of 20 nm. The demand for labeling density is even higher when 3D imaging is involved. Such a high demand for labeling density requires careful consideration and optimization of sample preparation and labeling methods. In practice, finite labeling density often becomes a major limitation in imaging resolution, especially for LBM, which achieves the highest resolution in biological samples among the super-resolution imaging modalities.

Analogous to the labeling density, the sampling density for the final image also has to be high for subdiffraction imaging. In other words, the image pixel size has to be small to record high-resolution information. A four-fold increase in resolution would require a 16-fold increase in pixel density in the lateral dimensions and 64-fold in 3D. This increase in pixel density may significantly slow data acquisition and puts high demands on the limited photon budget because each pixel must still detect a high enough number of signal photons to achieve a sufficient signal-to-noise ratio, and it requires a certain time to collect the photons at a given light intensity.

LBM Methods

LBM methods such as PALM, fPALM, STORM, GSDIM (ground state depletion followed by individual molecule return) and others generate subdiffraction images by reconstructing locations of single molecules^{9–11,16,46–48} (Fig. 2). These methods separate molecule fluorescence in time to utilize the fact that if the PSF of individual molecules can be

visualized, then the center of the PSF (i.e., the location of the fluorophore) can be determined with high precision, typically ~10–20 nm. In order to separate individual PSFs within a densely labeled sample, LBM methods use photoactivatable or reversibly photoswitchable fluorophores that are dark until being activated by short-wavelength light or conventional fluorophores driven into a dark state^{48,49}. By controlling the rate of conversion, only a few molecules are switched to the bright state in any given image frame. When the activated molecules are bleached by imaging light, a second subset of molecules is activated and imaged. After many iterations, the precisely localized center positions of individual molecules are overlaid to generate a final high-resolution image.

Several variants of LBM have been demonstrated, including multicolor PALM/STORM, 3D PALM/STORM and live PALM^{50–59}. Furthermore, in single particle tracking PALM (sptPALM) and related techniques, individual protein molecules in live cells are tracked until each fluorophore bleaches to derive diffusion or transport characteristics^{60–63}. The rapid development of LBM methods has led to several detailed reviews on the topic^{4,16,16,46,64}, and LBM microscopes are commercially available from Leica, Nikon, Vutara, and Zeiss. Custom systems can also be set up relatively easily based on an inverted fluorescence microscope^{46,57,64}.

Resolution limits: fundamental and practical—The localization precision of individual fluorophores improves roughly in proportion to the square root of the number of photons collected from each molecule before bleaching. The collection of 1000 photons per molecule (mean photon counts from EosFPs that are often used in PALM) provides a localization precision of ~10 nm. Further improvement in localization precision beyond 10 nm is possible, but it requires addressing other practical factors such as the size of the fluorescent marker, variations among camera pixels and drift of the stage and/or the sample^{65,66}. Furthermore, it has become increasingly appreciated that the finite labeling density of the target protein is usually the major limit of imaging resolution in LBM^{67,68} (see Focus Box).

Practical Considerations—The instrumentation for LBM is relatively simple and inexpensive, typically requiring only an inverted microscope with a high NA objective, a few well-aligned lasers, and a highly-sensitive camera^{46,57,64}. Although EMCCD cameras are typically used, sCMOS detectors have recently been adapted for higher LBM imaging speed at the expense of slightly lower sensitivity and pixel-dependent noise corrections⁶⁹. All LBM methods require photoactivatable or photoswitchable fluorophores with a very high bright-dark contrast ratio. This is because high-resolution images require high labeling density (see Focus Box). At high labeling density, the weak dark-state fluorescence of molecules with a low contrast ratio can sum up to create unacceptable levels of background fluorescence, preventing the detection of single molecules. Currently, both fluorescent proteins and organic dyes with high contrast ratios exist, but the selection is limited and not all photoactivatable organic dyes are commercially available. However, it was recently discovered that many types of conventional organic dyes can become photoswitchable under certain imaging conditions^{48,49,70–72}. Because LBM methods rely on single molecule imaging, they are very sensitive to the light scattering and aberration associated with thick samples. For those samples, very thin sections under fixed conditions have to be prepared before imaging^{50,73,74}.

LBM data collection can be time-consuming: it can easily take 5–30 minutes to sequentially activate and image all of the fluorescent labels in a densely-labeled sample. Data analysis is also computationally intensive, often requiring a time comparable to data collection on personal computers. However, if the raw image data is processed during acquisition, image reconstructions can be completed in real time⁷⁵. The slow data acquisition speed and severe

bleaching associated with current LBM methods have limited their application in live cell imaging. Recently, several researchers have presented improved LBM data analysis methods to speed up data collection by relaxing the requirement that individual emitter molecules be completely spatially separated in the raw images^{76–79}. The improvement in speed is typically paid for with increased computation time for molecule localization and image reconstruction. Other methods have dispensed with localizing bright molecules altogether and instead calculate a putative distribution of molecules from intensity fluctuations in a sequence of fluorescence images^{80–83}. These methods are computationally rapid but the resolution gain is moderate (~2 fold) compared to other LBM methods. In contrast to most LBM methods, sptPALM is suitable for live cell imaging because its goal is not to create a subdiffraction image *per se*. Instead, sptPALM and related methods monitor the local diffusive or directed motion and dynamic clustering phenomena by tracking individual fluorophores with high localization precision. An important consideration for these tracking methods is the careful tuning of the rate at which fluorescent molecules appear and disappear, because molecule locations need to be well-separated in order to track individual molecules accurately from frame to frame.

LBM methods are unlike other microscopic methods in that they treat the sample as a collection of individual fluorescent labels. This unique approach together with LBM's high spatial resolution demands rigorous data analysis strategies different from those for other types of fluorescent microscopy. Recently, several critical aspects in LBM data analysis have been addressed, including post-hoc assessment of image resolution^{67,68}, two-color colocalization^{84,85} and clustering^{86–88}. Notably, quantitative assessment of image resolution is an integral part of LBM image analysis because the achieved resolution is highly sensitive to experimental parameters and analysis methods. Similarly, claims of image resolution for novel techniques should be assessed quantitatively to facilitate direct comparison to existing methods.

General considerations and challenges for subdiffraction imaging

It is often underappreciated that regardless of the resolution achieved by subdiffraction imaging methods, any microscopic image can only be as good as the sample allows. Here, we will discuss several important factors and challenges for subdiffraction imaging methods.

Fluorophores and photon budget

The number of emitted photons required to achieve a sufficient signal-to-noise ratio is proportional to the number of pixels in the image, but with improved resolution of subdiffraction imaging, the required number of pixels increases in proportion to the square or cube (for 2D or 3D imaging, respectively) of the resolution improvement (See Focus Box). However, in any sample there is a finite number of photons available before all of the fluorophores are bleached. This limited “photon budget” is an important determinant of the achievable resolution in fluorescence microscopy.

Although the photon budget plays a slightly different role in each subdiffraction imaging modality, its effect is always to limit the amount of information available in the sample. In SIM, each subdiffraction image requires the capture of ~15 diffraction limited images and NL-SIM requires even more images at saturating light conditions. Each diffraction limited image collected reduces the amount of signal available in the photon budget for subsequent images. In STED/RESOLFT, most fluorophore excitation/activation events are quenched without producing signal photons, but each of these unproductive excitation/activation events may also lead to photobleaching, thereby reducing the fraction of the photon budget actually used for subdiffraction imaging. For both SIM and STED/RESOLFT, bleaching is more severe in 3D imaging because the excitation light for imaging one plane also bleaches

fluorophores at other depths (with the exception of Bessel SR-SIM²⁶). SIM and STED/RESOLFT will therefore both benefit from further developments in fluorophores and imaging conditions more resistant to bleaching. In the case of RESLOFT, photoswitchable fluorophores with faster on-off kinetics will greatly improve imaging speed as every pixel requires a full cycle of fluorophore switching before continuing the raster scan.

LBM methods are more efficient in utilizing the limited photon budget, since all the detected photons are used to localize individual molecules, but LBM methods would also benefit from fluorophore developments. As most fluorescent proteins used by PALM emit a median of less than 400 photons before bleaching, photoactivatable fluorescent proteins with increased photon counts per molecule may improve localization precision. In sptPALM and similar applications, where long-term tracking of a molecule is required, an increase in the photon counts also allows for longer single molecule trajectories. The contrast ratio of many photoswitchable fluorescent proteins and organic dyes, which limits the highest achievable Nyquist resolution (see Focus Box), can also be improved. Finally, both LBM and STED/RESLOFT methods, can benefit from further developments to expand the currently limited choices of fluorophores for multi-color imaging⁸⁹.

Labeling methods

The fluorescent labeling methods, whether exogenous (e.g., with antibodies) or endogenous (e.g., with FP tagging), require special consideration for subdiffraction imaging. Although antibodies exist for many targets, their affinity and specificity often need to be examined for superresolution imaging (see reference 90 for an example). Furthermore, the bulky size of antibodies (~25 nm if both primary and secondary antibodies are used) limits penetration into tissues and sets an upper spatial constraint for the labeling density, which in turn limits the achievable Nyquist resolution. The antibody size also introduces significant uncertainty in the localization of a protein target. Finally, antibody labeling is not usually compatible with live imaging of intracellular molecules. Therefore, challenges remain to develop highly specific antibodies and smaller alternatives such as single-chain antibodies⁹¹ and nanobodies⁹². These alternatives have further advantages in that they are single polypeptide proteins and can be expressed intracellularly, making them compatible with live cell imaging via the expression of a fluorescent protein tagged version within a cell^{93,94}. However, achieving stoichiometric labeling remains difficult and excess labels can be mislocalized.

On the other hand, FPs can be readily attached to most proteins stoichiometrically by molecular cloning methods with 100% specificity, and their small sizes (~2 nm) allow for high packing density and localization precision. In most experiments, FP-tagged protein is overexpressed within a cell, making it naturally compatible with live imaging. However, overexpression can lead to undesired perturbations in cell function and cause artifacts in protein localization (see reference 95 for an example). In principle, side effects of overexpression can be overcome by gene-replacement techniques, but typical knock-in strategies lead to the FP tag expressed in all cells where the target protein is typically expressed, resulting in a loss of imaging contrast in tissue. A current direction of research is to develop conditional knock-in strategies that allow the FP-tagged protein expressed at endogenous levels in a sparse subset of cells.

Sample preparation methods

It is not trivial to prepare a fixed biological sample so that both the labeling density and the molecular localization are appropriately maintained for subdiffraction imaging. Milder fixation conditions are often preferred for conventional fluorescence microscopy because strong fixatives, such as glutaraldehyde, can directly crosslink with proteins and reduce the

antigenicity of antibody epitopes. However, mild fixation conditions may lead to alterations in the subcellular structure and protein localization that, while not apparent with diffraction limited techniques, are visible under subdiffractive imaging conditions. Fortunately, our unpublished results and those of others⁹⁶ have indicated that many fluorescent proteins, such as the EosFP used in PALM, are resistant to even high concentrations of glutaraldehyde (2%), allowing the fixative to be used as part of sample preparation for PALM. Overall, optimization of sample preparation conditions is usually required to achieve a balance between the preservation of the cellular architecture and the labeling density for subdiffractive imaging.

A further consideration for LBM methods is drift of the sample during lengthy data acquisitions. A drift as small as 10 nm/minute can drastically distort subdiffractive structures when single molecule locations are collected over 10s of minutes. Fortunately, this drift can be corrected if a few small, fluorescent fiducials are added to the sample. Fiducials, such as gold beads that are resistant to photodamage and are fluorescent in both red and green channels, can also be used for precise, post-hoc registration of two color images.

A currently active area of research is the adaptation of EM sample preparation methods to produce resin-embedded ultrathin sections for subdiffractive imaging^{73,74,96}. Such an approach has many advantages: it improves the z resolution since most ultrathin sections are less than 100 nm, it eliminates most of background fluorescence and light aberration that are associated with thicker samples, and it usually achieves a high degree of structural preservation. It is important to note that subdiffractive imaging can reveal previous unknown details that are difficult to interpret on their own. Using ultrathin sections with transmission or scanning EM, it may be possible to correlate the subdiffractive fluorescence images with the ultrastructural cellular context of the same sections^{73,74}. Such LBM and EM correlation has significant advantages over immuno-EM because it can achieve much higher labeling density. When fully developed, the correlation of EM with LBM or other subdiffractive imaging methods has the potential to revolutionize our understanding of protein organization at the ultrastructural level.

Real-World Application of Subdiffractive Imaging and Biological Results

Although challenges remain in subdiffractive imaging, researchers have begun to apply these powerful methods to examine biological questions. Many important discoveries have been made, and several areas of study have been approached by multiple classes of subdiffractive imaging. Here, we summarize biological results organized by topic.

Neuroscience

The billions of neurons in our brain are wired together via trillions of tiny synapses, most of which are smaller than 1 μm . Each synapse contains finely organized subregions and small structures, such as presynaptic vesicles, the presynaptic active zone, and the postsynaptic density. The organization at and within these sub-synaptic structures is thought to play an important role in shaping synaptic function. However, the details of these sub-synaptic structures are largely lost in diffraction-limited imaging.

Among the subdiffractive microscopic modalities, LBM probably provides the best access for measuring synaptic protein organization because its resolution approaches the length scale of subsynaptic structures (~ 10 nm). To this end, Dani et al.⁵⁰ imaged pairs of pre- and post-synaptic proteins across many synapses in antibody-labeled thin sections of mouse cortex using two-color 3D STORM imaging. Their systematic approach resulted in a schematic of protein localization relative to the synaptic cleft (Fig. 3A). By imaging antibodies targeted at different epitopes of the same protein, the authors were also able to

determine the orientation of two presynaptic proteins, piccolo and bassoon. The use of epitope-specific antibodies or N- and C-terminal fusion proteins to uncover protein orientation is an important strength of subdiffraction imaging that has also been applied to the analysis of other protein complexes (see below). In addition, Macgillavry et al. also used LBM methods to reveal the local heterogeneity within individual postsynaptic densities⁹⁷.

LBM techniques have also allowed live visualization of protein dynamics in cultured neurons. Frost et al. used sptPALM methods to identify discrete regions of actin polymerization in dendritic spines, the tiny protrusions on dendritic shafts that receive most excitatory synaptic inputs (Fig. 3B)⁶¹. Similarly, sptPALM has allowed direct measurement of the dynamics of glutamate receptors in cultured neurons showing dynamic clustering of receptors within spines⁹⁸, and directed flow of AMPA-type glutamate receptors in or out of individual spines⁶² (Fig. 3C).

We have employed sptPALM to examine the mechanism underlying signaling compartmentalization in neurons. Many neuronal signaling events appear to be compartmentalized at the level of individual synapses, despite the known mobility of the involved signaling molecules. As synapses are very small, protein diffusion should have a profound effect on signaling molecule dynamics and localization. It is poorly understood how signaling compartmentalization is maintained under these conditions. To this end, we have examined the diffusion of the catalytic subunit of protein kinase A (PKAc) in postsynaptic spines. PKAc, upon activation, is released from the immobile regulatory subunits to diffuse and act on its substrates. Our sptPALM data showed that the change in PKAc mobility upon PKA activation can be extracted from single molecule trajectories of EosFP-tagged PKAc (Fig. 3D). Since PKAc's catalytic activity is directly related to its mobility, sptPALM can provide a readout of local PKA activity with subdiffraction resolution.

An exciting aspect of the development of subdiffraction imaging is the discovery of entirely new phenomena and structures. One example is the recent description of a periodic cytoskeletal structure encircling axons in the mammalian central nervous system (Fig. 3E)⁹⁹. Although the periodicity of this structure (~200nm) is large by subdiffraction imaging standards, it is below the diffraction limit and unable to be resolved by conventional fluorescence methods. On the other hand, this structure is large enough and sparse enough that it has not been reported in EM images either. Although the function of these periodic structures is not yet known, this result illustrates that there are unknown cellular structures to be discovered below the diffraction limit.

LBM encounters great difficulties for studies in living brain tissue, because severe light scattering and aberration in tissue prevents the detection of single molecule fluorescence. However, superresolution spine morphology and dynamics in brain slice have been documented using STED¹⁰⁰, 2-photon STED variants^{34–36} and RESOLFT¹⁰¹. STED has also been employed to examine the dynamics of membrane proteins in neurons during exocytosis¹⁰². Although most results involving STED were obtained with custom systems, they illustrate an important advantage of STED-related techniques for neuroscience: imaging relatively deep within live tissue (Fig. 3F).

Eukaryotic cell biology and protein signaling complexes

Macromolecular protein structures play critical roles in signal integration and downstream action in cells. In recent years, subdiffraction imaging has been used to reveal the detailed features of several large protein complexes. Focal adhesion complexes link a cell's cytoskeleton to the extracellular matrix, enabling force generation, and sensing the local mechanical and chemical environment. Analysis of protein location and function within

focal adhesions is nearly impossible with conventional light microscopy because the structures are small, typically ~100 nm across, and occupy a very thin region near the substrate. Using a custom-built interferometric PALM (iPALM) microscope that can achieve a z resolution of less than 10 nm (in addition to resolution improvements in the x-y plane⁵¹), Kanchanawong et al. deciphered the arrangement of 9 focal adhesion and cytoskeletal proteins, yielding the first schematic of focal adhesion organization derived from direct measurement *in situ* (Fig. 4A)³¹. The authors also utilized N- and C-terminal FP fusions of the actin binding protein talin to determine its orientation relative to the larger focal adhesion structure. The lateral organization of a related subcellular structure, the podosome, was recently investigated using 2D STORM, resulting in a similarly detailed model of its components¹⁰³.

The centrosome is an enigmatic organelle with dimensions near the diffraction limit that has recently been investigated using subdiffraction imaging. The centrosome is present at the spindle poles of nearly all eukaryotic cells during mitosis^{104,105}, and is made of two centrioles and a cloud of peri-centriolar material (PCM) that appears amorphous in conventional fluorescence images. Both SIM and STORM have been used to determine the location of centrosomal proteins in *Drosophila* (Fig. 4B)^{106,107} and in a human cell line¹⁰⁸, including revealing organized protein distributions within the PCM, which was previously thought to be disordered. The relatively high number of proteins screened in these studies (7, 14 and 18 in references 106, 107 and 108 respectively) indicates that subdiffraction imaging is able to efficiently dissect the spatial relationships between proteins within large macromolecular complexes. Centrioles are also present at the base of cilia, and some epithelial cells can be cultured under conditions where hundreds of centrioles are all oriented perpendicular to the imaging plane. Lau et al. took advantage of this geometry using STED microscopy and optimized immunolabeling methods to directly observe a 9-fold symmetry of the centriolar protein Sep164 (Fig. 4C)¹⁰⁹. In addition to its central biological finding, this study illustrates the aforementioned challenge for subdiffraction imaging: improvements in labeling protocols or other sample preparation methods may be required to achieve the full potential of each method.

Another protein structure being explored with subdiffraction imaging is the nuclear pore complex (NPC). An early SIM study examined the DAPI-stained DNA in the nucleus, as well as immunolabeled nuclear lamina and the NPC. This study resolved individual NPCs, observed invaginations in the nuclear membrane that were previously only visible under EM, and showed that chromatin was excluded from the neighborhood of the NPC (Fig. 4D)²³. The structure of the NPC was further investigated in *Xenopus laevis* oocyte nuclear envelopes using dSTORM by Löschberger et al.¹¹⁰, revealing eightfold symmetry of NPC protein gpc210 and measuring the NPC diameter with 7 nm precision. Also, Chatel et al. used SIM to confirm the relative radial location of four nucleoporin proteins within the NPC that were previously estimated from immuno-EM measurements¹¹¹. Very recently, Szymborska et al. combined STORM imaging with particle-averaging techniques to precisely measure the radial position of six NPC proteins (Fig. 4E)¹¹². The authors integrated their precise position measurements with nanobody labeling of N- and C-terminal FP-fusion proteins to reconstruct the location and orientation of these proteins from thousands of subdiffraction NPC images. For structures like the NPC and centriole, which have a relatively simple geometry, the employment of particle averaging methods may enhance the ability of subdiffraction imaging to decipher the structure of macromolecular complexes.

Microbiology

Bacteria were often considered to have little subcellular organization, but studies in recent decades have shown that prokaryotes possess intricate internal structure, including cytoskeletal elements, active transport machinery for cell division and other specialized macromolecular structures (e.g., see references 113 and 114). Given that many prokaryotic cells are only a few microns in size, visualizing and understanding bacterial substructure presents a nearly insurmountable challenge for conventional fluorescence microscopy. In this context, subdiffractive imaging has provided new insight into the inner workings of bacteria.

One critical element of the bacterial cytoskeleton, FtsZ, is part of the contractile structure called the Z-ring, which is responsible for cell division. The localization of FtsZ to the Z-ring can be observed with conventional microscopy, but further details have been obscured by the diffraction limit. Using sptPALM, Niu and Yu demonstrated in *E. coli* that, while FtsZ in the Z-ring was immobile and likely polymerized, FtsZ molecules far away from the Z-ring were mobile and distributed non-homogeneously throughout the cell¹¹⁵. Following this study, further PALM investigations in fixed and live *E. coli* cells showed that the Z-ring is comprised of short, loosely grouped FtsZ filaments that can form a variety of helical structures near the midline of the cell¹¹⁶. FtsZ monomers were also observed to turn over rapidly (~100ms) in the Z-ring of *C. crescentus*¹¹⁷. At a resolution lower than LBM, 3D SIM offers complementary, live-cell imaging of the entire Z-ring in *S. aureus* and *B. subtilis* to yield a few key results: 1) the amount of FtsZ in the Z-ring does not depend on the Z-ring diameter, and 2) FtsZ, along with Z-ring proteins PBP2 and EzrA, forms an inhomogeneous distribution around the z-ring, including dynamic gaps and clusters¹¹⁸ (Fig. 4F).

Repair of damaged DNA is a critical cellular process that prevents propagation of potentially harmful mutations. Although the biochemical reaction rates of numerous DNA polymerization and ligation enzymes involved in DNA repair have been determined *in vitro*, little is known about the cell-to-cell variability of DNA repair processes and the dynamics of these same enzymes in the cellular environment. Recent work has utilized sptPALM measurements to tease apart the reaction rates, diffusion speeds and the spatial arrangement of reaction sites for DNA polymerase I and ligase from single molecules in individual *E. coli* cells¹¹⁹. In response to DNA damage, the amount of time spent by these repair enzymes bound to DNA increases and the total number of DNA repair events was quantified directly from the accumulated single molecule trajectories. These experiments represent a future direction for a wide range of biological research, whereby biochemical reaction rates can be directly measured in live cells on a single molecule basis.

Summary and Future Directions

The recent progress in subdiffractive microscopy has started to allow biologists to investigate subcellular architecture and molecular dynamics at high-resolution. These superresolution techniques have described previously unknown structures, clarified macromolecular complex organization, and revealed dynamics of critical synaptic proteins. The gains in microscopic resolution made possible by SIM, STED, LBM, and their related techniques present new demands and challenges in fluorophore development, labeling methods, and sample preparation. As the field advances, increased throughput and efficiency of image acquisition and data analysis will allow the investigation of a large number of proteins and structures particularly relevant to systems biology. Although challenges and limitations remain, subdiffractive microscopy is rapidly becoming a fundamental tool for biological research.

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Figure 1. The need for subdiffraction imaging and current commercially available subdiffraction approaches

A) Cartoon of two point sources of light separated by 150 nm and their overlapping PSFs (approximated by Gaussians) typical of confocal microscopy. Approximated PSF in the x-z plane is shown on the right at the same scale. **B)** Diffraction limited PSF overlaid to scale on an EM image of a dendritic spine, illustrating the difficulty of diffraction-limited microscopy in localizing synaptic proteins. **C)** The number of publications per year citing the use of each subdiffraction method (see Note for search terms). **D)** Appearance of two point sources separated by 150 nm via current commercially available subdiffraction microscopy methods. The PSF cartoons represent the effective precision associated with each method.

Note: Data for Figure 1C were collected from scopus.com and limited to articles in journals using the search terms below. Data are plotted through 2012.

Search terms (ar = article, j = journal)

SIM: (TITLE-ABS-KEY("structured illumination microscopy") OR TITLE-ABS-KEY("structured-illumination microscopy")) AND (LIMIT-TO(DOCTYPE, "ar")) AND (LIMIT-TO(SRCTYPE, "j"))

STED: ((TITLE-ABS-KEY("stimulated-emission depletion" AND microscopy) OR TITLE-ABS-KEY("stimulated emission depletion microscopy") OR TITLE-ABS-KEY("stimulated-emission-depletion fluorescence microscopy") OR TITLE-ABS-KEY("stimulated emission depletion nanoscopy") OR TITLE-ABS-KEY("RESOLFT") OR

TITLE-ABS-KEY("stimulated emission depletion fluorescence nanoscopy")) AND
(LIMIT-TO(DOCTYPE,"ar")) AND (LIMIT-TO(SRCTYPE,"j")))
LBM: ((TITLE-ABS-KEY("stochastic optical reconstruction microscopy") OR TITLE-
ABS-KEY("photoactivated localization microscopy") OR TITLE-ABS-
KEY("photoactivation localization microscopy") OR TITLE-ABS-KEY("fluorescence
photoactivation localization microscopy") OR TITLE-ABS-KEY("fluorescence
photoactivated localization microscopy") OR TITLE-ABS-KEY("photoactivation-
localization microscopy")) AND (LIMIT-TO(DOCTYPE,"ar")) AND (LIMIT-
TO(DOCTYPE,"ar")) AND (LIMIT-TO(SRCTYPE,"j")))

Figure 2. The principles and representative images of the subdiffractive microscopic methods
 Scale bars are 4 μm . Images in the top panel (actin in Hela cell) is used with permission⁷ and middle panel images (actin in embryonic chick fibroblast) are courtesy of Elise Stanley (Toronto Western Research Institute and University of Toronto). Bottom images (actin tagged with mEos3.2 in fox lung fibroblast) are our unpublished work.

Figure 3. Studies utilizing subdiffractional microscopic methods in neuroscience

A) The subsynaptic localization of both pre- and post-synaptic proteins examined using STORM⁵⁰. **B)** sptPALM was used to track actin and identify discrete regions of actin polymerization in dendritic spines⁶¹. **C)** sptPALM measured AMPA receptor dynamics in cultured neurons⁶². **D)** sptPALM data showed that the change in PKAc mobility upon increased cAMP concentration can be extracted from single molecule trajectories of PAFP-tagged PKAc. (BRL and HZ, unpublished) **E)** STORM revealed a previously unobserved periodic ring structure, containing actin, adducin and spectrin in the axons of cultured neurons⁹⁹. **F)** *in vivo* STED images showing the dynamic nature of EYFP labeled spines at high resolution, 10–15 μm below the surface of the cortex⁴¹. All images used with permission from their respective publishers.

Figure 4. Subdiffraction imaging of protein complexes in eukaryote cell biology and microbiology

A) An iPALM system was used to map the spatial distribution of focal adhesion associated proteins in the z dimension⁵¹. **B)** SIM was used to examine multiple centrosomal proteins. The images of each protein were aligned and averaged (left), and mapped to the center of the centriole (right)¹⁰⁷. **C)** STED microscopy combined with optimized sample preparation and antibody labeling showed that the centriolar protein cep164 makes a nine-cluster ring that can not be seen with confocal microscopy¹⁰⁹. **D)** SIM was used to map the arrangement of nuclear pore associated proteins²³. **E)** SIM was combined with particle averaging analysis to determine the organization of the y-shaped Nup107-160 complex (left) at the nuclear pore, which was further mapped onto cryo-EM structure of the pore complex (center, right)¹¹². **F)** Live 3D SIM was used to track bacterial Z-ring proteins and show that they form dynamic clusters¹¹⁸. All figures reproduced with permission from their respective publishers.