Spectroscopic analysis beyond the diffraction limit

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Abstract

The recent surge in spectroscopic Single-Molecule Localization Microscopy (sSMLM) offers exciting new capabilities for combining single molecule imaging and spectroscopic analysis. Through the synergistic integration of super-resolution optical microscopy and single-molecule spectroscopy, sSMLM offers combined strengths from both fields. By capturing the full spectra of single molecule fluorescent emissions, sSMLM can distinguish minute spectroscopic variations from individual fluorescent molecules while preserving nanoscopic spatial localization precision. It can significantly extend the coding space for multi-molecule super-resolution imaging. Furthermore, it has the potential to detect spectroscopic variations in fluorescence emission associated with molecular interactions, which further enables probing local chemical and biochemical inhomogeneities of the nano-environments. In this review, we seek to explain the working principle of sSMLM technologies and the status of sSMLM techniques towards new super-resolution imaging applications.

Keywords: Super-resolution microscopy; Fluorescence spectral imaging; Single-molecule spectroscopy

1. Introduction

In the past decade, a wide range of super-resolution imaging modalities, including structured illumination microscopy (SIM), stimulated emission depletion microscopy (STED), and single-molecule localization microscopy (SMLM), have been developed to improve the optical imaging resolutions far beyond the diffraction limit of light (Hell, 2007; Huang et al., 2010; Liu et al., 2015; Moerner, 2015). These techniques have offered tremendous opportunities for researchers to "see" cellular architectures at single-nanometer scale, which was previously considered unresolvable by optical microscopy. However, despite the successes in precisely capturing the spatial locations of individual fluorescence molecules, the fluorescent emission information were only detected by rather limited color channels, leaving the rich molecular spectroscopic signatures largely un-utilized.

To address the above-mentioned difficulties, multiple research groups recently reported a new class of imaging technology based on the working principle of SMLM that simultaneously recorded the spatial distributions and the associated spectral signatures of stochastic fluorescence emissions from individual

fluorescent dye molecules (Dong et al., 2016a; Mlodzianoski et al., 2016; Zhang et al., 2015). Here we refer to this class of imaging technology as spectroscopic Single-Molecule Localization Microscopy or sSMLM. By capturing and analyzing the full emission spectra of single-molecule emission events, sSMLM is capable of distinguishing their minute spectroscopic variations. Thus, it can significantly increase the number of different fluorescence labels that can be imaged simultaneously as compared with existing multi-color super-resolution microscopy methods using discrete color channels (Dong et al., 2016a; Mlodzianoski et al., 2016; Zhang et al., 2015). Moreover, the combination of the nanoscopic spatial resolution and the fine spectra resolution further enables highly sensitive measurement of spectroscopic variations due to potential molecular interactions to probe the chemical and biochemical variations of the microenvironments (Bongiovanni et al., 2016; Dong et al., 2017; Moon et al., 2017). In this review, we will first explain the working principle of sSMLM technologies and then discuss their applications to super-resolution multi-molecular and functional imaging.

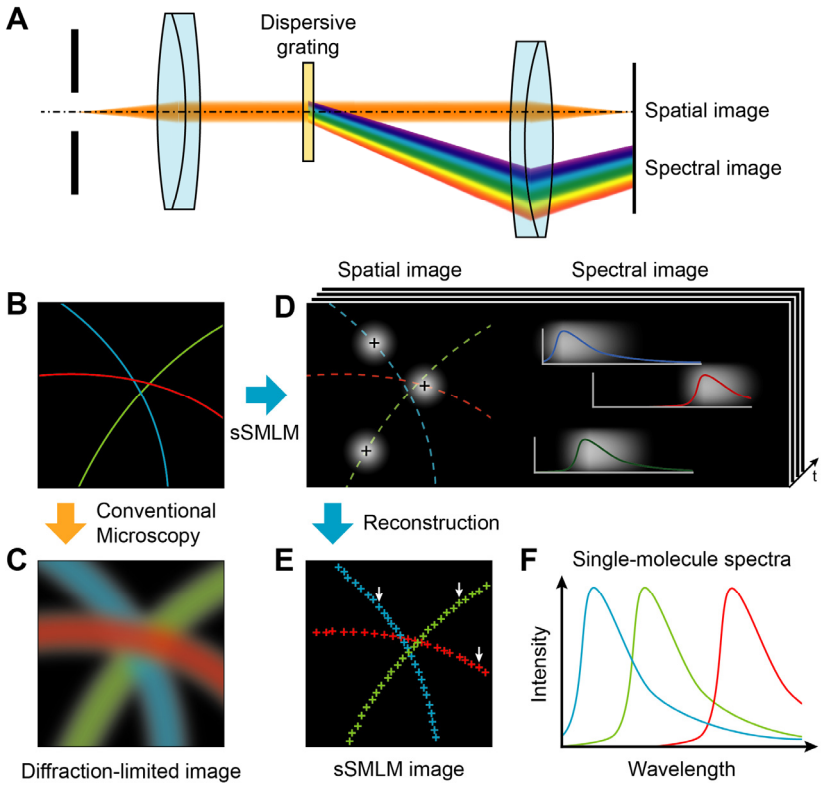

Figure 1. Working principle of sSMLM. (A) A reported sSMLM system using a dispersive grating to form spatial and spectral images on the same array detector (Dong et al., 2017). (B) An illustration of three fibrous structures consisting of different types of molecules and hypothetically emitting distinct colors. (C) The corresponding diffraction-limited image of the three colored fibers obtained by conventional microscopy. (D) A movie containing a sequence of sSMLM images. A representative frame shows three stochastic radiation events and their corresponding emission spectra was acquired by a camera. (E) Reconstructed color-coded super-resolution image. (F) Illustration of single-molecule spectra of radiation events denoted by arrows were extracted simultaneously.

2. Working principle

It is well-known that SMLM correlates each stochastic radiation event of individual fluorescent molecules to the probabilistic locations from their point spread functions, thus, providing sub-diffraction-limited spatial resolution. Building upon the same working principle, sSMLM incorporates an additional dispersive element to capture the spectroscopic information. Specifically, prisms are used in the reported system on spectrally-resolved stochastic optical reconstruction microscope (SR-STORM) (Moon et al., 2017; Zhang et al., 2015) and spectral fluorescence photoactivation localization microscopy (Mlodzianoski et al., 2016). Meanwhile, blazed diffraction gratings were used in other implementations of sSMLM, such as spectroscopic photon localization microscopy (SPLM) (Dong et al., 2016a; Dong et al., 2017; Urban et al., 2016) and spectrally-resolved points accumulation for imaging in nanoscale topography (sPAINT) (Bongiovanni et al., 2016).

In Fig. 1, we use a conceptual model to explain the general working principle of sSMLM. Fig. 1A illustrates a reported sSMLM system using a dispersive grating (Dong et al., 2017). The stochastic fluorescence emission from a single molecule, which contains a burst of photons, is collected and further divided to form spatial and spectral images. The photons allocated to the spectral image pass through the dispersive element to reveal the fluorescence spectra of individual fluorescence dye molecules. The remaining photons are allocated for the spatial image that does not impose additional dispersive characteristics. As shown in Fig. 1B, a hypothetical example of fibers consisting three different molecules each with a distinct emission spectrum. When imaged with conventional optical microscopy, the nanoscopic feature formed by the three fibers cannot be revealed due to optical diffraction limit (Fig. 1C). As molecules are rapidly switched between ‘on’ and ‘off’ states, an electron multiplying charge-coupled device (EMCCD) camera or a scientific complementary metal-oxide-semiconductor (sCMOS) camera records a movie containing a time sequence of both spatial and spectral images (Fig. 1D). The spatial image is used to localize the positions of individual stochastic radiation events from a single molecule and to provide the inherent reference location for the spectral analysis (Fig. 1E). The procedure to process the spatial image is identical to conventional SMLM method. For each stochastic emission event, well-establish methods (Mortensen et al., 2010), such as the two-dimensional Gaussian fitting, can be used to determine the spatial location of the single-molecular emitter. In order to convert the recorded spectral images in the spatial domain to the emission spectrum in the spectral domain, a spectral calibration procedure is required to establish the spatial-wavelength mapping, which is specific to the configuration of the sSMLM system (Dong et al., 2016a; Mlodzianoski et al., 2016; Zhang et al., 2015). Finally, the spectral image can be analyzed to extract the emission spectrum of the single-molecular emitter based on the spectral calibration (Fig. 1F).

Two main configurations of sSMLM differ in the use of either refraction prisms or diffraction gratings as the dispersive components with matching optical components and image analysis algorithms. In the sSMLM system using a refraction prism, the fluorescence emissions are first separated to follow two optical paths using a beam splitter with a desired splitting ratio. One optical path is dedicated to generate the spatial image for localization reconstruction. The other optical path incorporates a refraction prism to generate the spectral image. The refraction prism has relatively low dispersion, which is more suitable for applications requiring low spectral precision. However, correcting the inherent nonlinear spectral dispersion, which is characteristic of refraction prisms, requires a sophisticated calibration process. Additionally, the optical loss occurs at surfaces of the beam splitter, the prism, and reflection mirrors used in this configuration. A carefully designed broadband anti-reflection coating is needed to minimize the optical loss. In contrast, in the sSMLM system using a diffraction grating, the spatial images and spectral images can be obtained directly by collecting photons in the 0^{th} and the 1^{st} diffraction order, respectively. Compared to the prism-based sSMLM system, the grating-based sSMLM system eliminates the needs for creating two separated optical paths and simplifies the optical system configuration. To be noted, the grating efficiency is wavelength dependent based on the characteristic of the grating and needs to be calibrated experimentally. For example, for a blazed transmission grating with a blaze wavelength of 580 nm, the grating efficiency within 500-700 nm is around 17-30% at the 0th order and 54-68% at the 1st order, which leads to approximately 15% of total energy loss to other diffraction orders. In contrast to prisms, gratings provide a much wider range of spectral dispersion with improved linearity and are more suited for applications requiring high spectral dispersions and quantitative analysis. Moreover, using gratings can avoid sophisticated optical alignment, making the system more compact and compatible with existing SMLM systems, and offers potential adjustable spectral dispersion by switching gratings without further optical alignment. In principle, the two approaches can provide similar performances for sSMLM and the optimal solution depends on the specific application.

To be noted, the number of photons from single stochastic radiation events remains the limiting factor of the sSMLM. As sSMLM allocates a portion of the photons to form the spectral image, it unfavorably reduces the number of photons for the spatial image and, consequently, the localization precision. Moreover, overlapping of spectra among multiple single-molecule emission events also introduces a restriction in the maximum fluorophore density that can be imaged with sSMLM and, therefore, limits the imaging speed. To increase the maximum fluorophore density, a modified linear unmixing algorithm has been developed to separate overlapped spectra when single emitters are well separated in the spatial image (Dong et al., 2016a). However, in contrast to conventional SMLM, multi-emitter analysis methods are not yet available in sSMLM as spectra of individual overlapped emitters cannot be separated.

3. Super-resolution spectral imaging

Using the full emission spectra to discern the signatures of different fluorescent dye molecules represents a methodological advancement over the commonly used multicolor imaging techniques, such as the ratiometric method (Gunewardene et al., 2011). In the ratiometric method, fluorescence emission of each molecule was split into multiple color channels and identified by the ratiometric comparison of intensities in each channels. This method is limited in the maximum number of fluorescent species simultaneously imaged, which is fundamentally limited by the inherent spectral heterogeneity of fluorescent probes and further constrained by the limited signal-to-noise ratio. In contrast, simultaneously characterizing multiple dye molecules with their full spectroscopic information largely extends the combination of discernible markers and improves imaging speed in multi-labeled samples. Given a sample labeled with multiple fluorophores with peak emission wavelength separations of ~20 nm, it is theoretically possible to simultaneously image up to 15-20 different fluorescent species (Mlodzianoski et al., 2016), suggesting that sSMLM could provide much greater potential for imaging complex biological processes simultaneously. However, generation of a sample with a large number of different fluorescent labels remains challenging. To date, the maximum number of fluorescent labels demonstrated experimentally is four (Zhang et al., 2015), as shown in Fig. 2A. In addition, sSMLM provides the capability to identify imaging artifacts through their distinct emission spectra (Dong et al., 2016a). Recent cellular studies using sSMLM revealed scattered localized events, which showed distinct emission spectra other than those of the dye molecules used to label the sample (Dong et al., 2016a). Those localized events were most likely from endogenous autofluorescence (Dong et al., 2016b; Urban et al., 2016) or from unknown sources of fluorescence induced by, for example, the use of fixatives or DNA transfection reagents (Whelan and Bell, 2015). This phenomenon is often overlooked by conventional SMLM and may be incorrectly attributed to the nonspecific antibody or probe binding (Whelan and Bell, 2015). Currently, sSMLM provides the capabilities to identify such imaging artifacts and will help to develop a better understanding of their physical origins.

Additionally, it has been demonstrated that sSMLM can be used to distinguish minor differences in fluorescent spectra, allowing identification of individual molecules, even among the same type of molecules (Dong et al., 2016a). It has been observed that the emission spectra of single fluorophores can vary due to their conformational variations and interactions with the heterogeneous local environment (Ambrose and Moerner, 1991; Trautman et al., 1994). For example, the experimentally recorded spectral heterogeneity of Rhodamine molecules has a full width at half maximum of ~20 nm in their spectral centroid distribution (Fig. 2B). By capturing the molecule-specific spectroscopic signatures, the origins of photon emissions from different molecules can be identified according to their spectral differences. As a result, the photons

emitted from the same molecule captured in different frames can be combined with molecule discrimination through spectral regression. Therefore the fundamental resolution limit of SMLM can be extended (Dong et al., 2016a). Fig. 2C shows the comparison of conventional SMLM image and SPLM image with spectral regression of two closely spaced microtubules (Dong et al., 2016a). A four-fold improvement in the spatial resolution was demonstrated in Rhodamine-labeled microtubules using SPLM with spectral regression (Dong et al., 2016a).

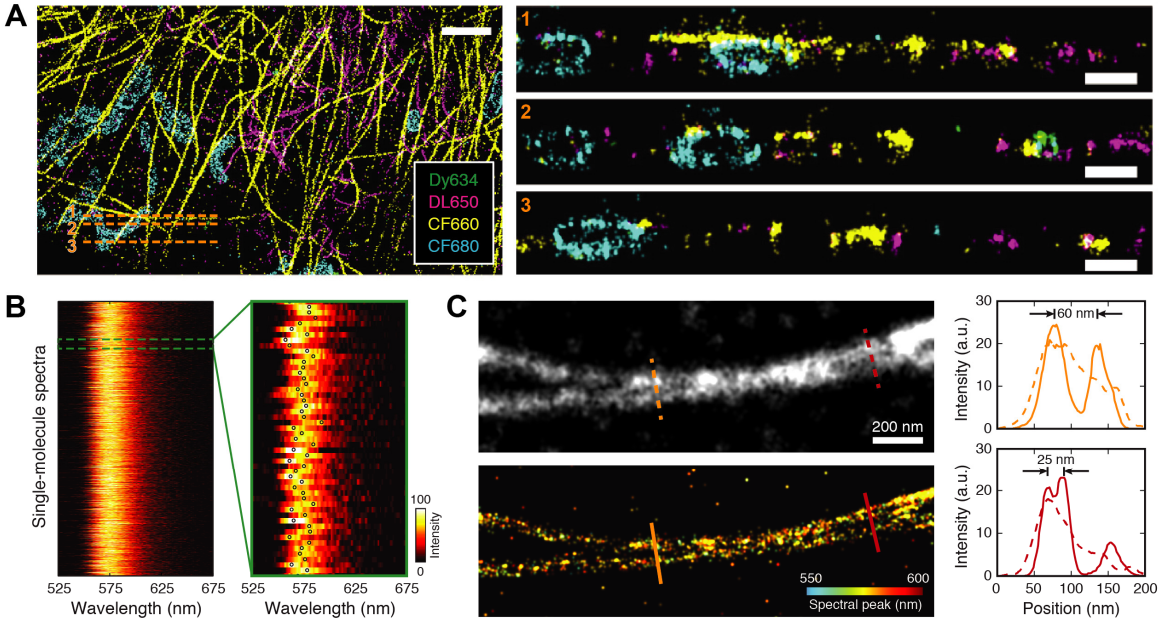

Figure 2. (A) 3D SR-STORM imaging of a fixed COS-7 cell (Zhang et al., 2015), where peroxisomes, vimentin filaments, microtubules, and the outer mitochondrial membrane were labeled by Dyomics 634, DyLight 650, CF660C, and CF680, respectively. Cross-sectional views along the three red-dashed lines. Scale bars: (left) 2 μm and (right) 500 nm. (B) Emission spectra along a single Rhodamine-labeled microtubule, which indicated the spectral heterogeneity even among the same type of dye molecules (Dong et al., 2016a). (C) Conventional SMLM image of two closely positioned microtubules and its corresponding SPLM image with spectral regression (Dong et al., 2016a). Line profiles from positions highlighted by the dashed and solid lines in the SMLM (top) and the SPLM (bottom) images indicate the improvement of imaging spatial resolution. Scale bar: 200 nm.

4. Super-resolution imaging of complex biological processes

sSMLM has also been demonstrated to enable functional super-resolution imaging of changes in the cellular nanoenvironment (Bongiovanni et al., 2016; Moon et al., 2017). For example, hydrophobicity or the repulsion for water molecules by various cellular membranes was investigated by quantifying the spectral heterogeneity using water sensitive dye Nile-Red as a hydrophobicity probe. Bongiovanni et al. studied differences in membrane hydrophobicity using 1-2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin (SM), and sphingomyelin mixed with cholesterol (SM+CL) vesicles (Bongiovanni et al., 2016). The DOPC bilayer is the least ordered membrane since its low hydrophobicity enables water

molecules to percolate across the membrane. The SM+CL vesicles had the most ordered membrane since SM+CL molecules were highly hydrophobic thus preventing the transport of water across the membrane. A decrease in hydrophobicity resulted in a red-shift of the fluorescence emission of Nile-Red demonstrated by the spectra of DOPC vesicles in comparison to SM+CL vesicles. Based on this finding, sSMLM was used to investigate changes in aggregates of proteins associated with Parkinson's and Alzheimer's (alpha-synuclein and amyloid-beta). Using this method, a large number of proteins can be screened and the changes in hydrophobicity can be related to toxicity and disease progression, as shown in Fig. 3A. A more comprehensive study was further developed to investigate the impact of cholesterol levels on membrane hydrophobicity (Moon et al., 2017) (Fig. 3B). Depleting cholesterol with methyl-beta-cyclodextrin (MβCD) led to a strong red-shift of Nile-Red single-molecule spectra of the plasma membrane (indicating a decrease in hydrophobicity) but little change of the spectra from organelle membranes. In contrast, after adding 1 mM water-soluble cholesterol (cholesterol−MβCD), the organelle membrane spectra blue-shifted significantly, becoming closer to that of the plasma membrane as the latter remained spectrally unchanged. These results indicate that cellular cholesterol level is responsible for differences in the hydrophobicity of plasma and organelle membranes, which can be observed in sSMLM using Nile-red as a functional probe. By tailoring the function of the fluorescent dye, sSMLM can be broadly applied to probe a wide variety of local chemical and biochemical homogeneities at deep nanometer scale.

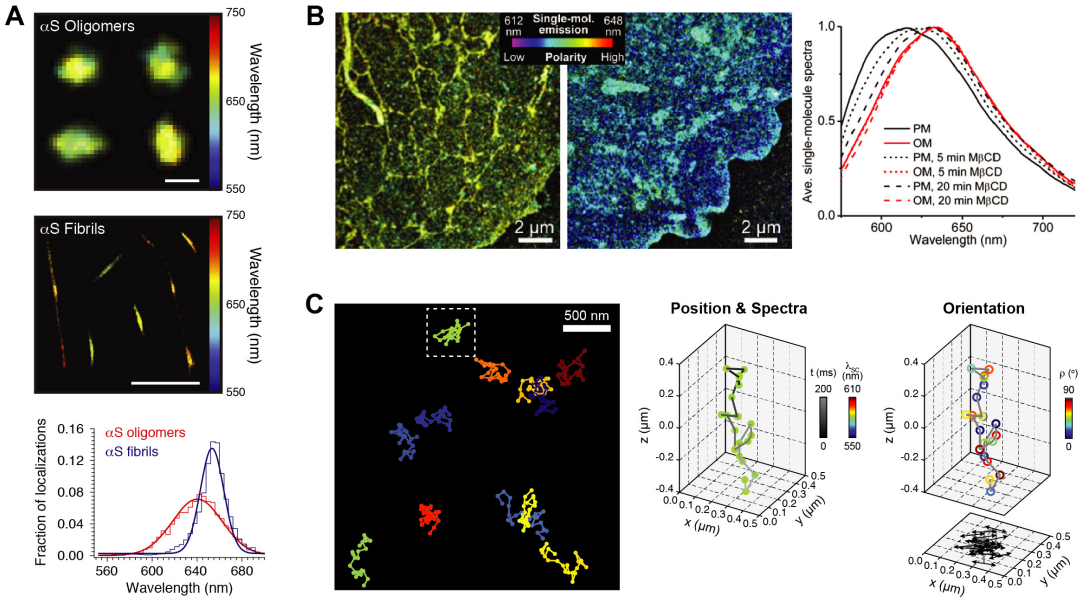

Figure 3. (A) sPAINT images of protein aggregates associated with disease (Bongiovanni et al., 2016). Histogram of the individual emission spectra from αS oligomers to αS fibrils indicates the red-shift corresponding to a decrease in hydrophobicity and the narrowing of the distributions during the fibril generation. (B) Observed heterogeneity in cellular membrane hydrophobicity is driven by cholesterol (Moon et al., 2017). SR-STORM images of a fixed COS-7 cell labeled with Nile Red indicate a spectral red-shift and blue-shift after depletion of cholesterol with 5 mM MβCD and after cholesterol enrichment with 1mM water-soluble cholesterol, respectively. (C) Multi-contrast parallel single

particle tracking using 3D-Polar-SPLM at a temporal resolution of 10 ms (Dong et al., 2017). Emission spectra and polarization angle with respect to its position in 3D of a single moving QR in the highlighted region were illustrated.

Furthermore, combining additional optical contrasts in sSMLM can provide a platform for unraveling nanoscale structures at the single-molecule level. For example, polarization anisotropy of fluorescence emission was commonly used to elucidate the structural organization of molecular assemblies (Backer et al., 2016; Cruz et al., 2016; Gould et al., 2008). In this regard, a three-dimensional (3D), polarization-sensitive, spectroscopic photon localization microscopy was developed to enable parallel 3D tracking of individual molecules and nanoparticles while simultaneously capturing their fluorescence spectra and polarization states with high precision (Dong et al., 2017) (Fig. 3C). While polarization states reflect the structural orientation of quantum rods (QRs), spectral profile of the fluorescence emission provided a particle-specific signature for identifying individual QRs among the heterogeneous population, which significantly improved the fidelity in parallel 3D tracking of multiple QRs. This approach can potentially be extended to synthetic fluorescence molecules widely used in biological imaging, adding strength to single-molecule imaging and spectroscopy (Kusumi et al., 2014). Future integration of additional parameters to realize the quantification of molecular interactions at higher multiplexing dimensions (using fluorescence intensity, lifetime, color, and polarization) is a promising approach to improve sensitivity, specificity, spatial resolution, and realize multiparameter detection.

5. Conclusion and perspectives

sSMLM can not only largely extend the number of discriminable fluorescent labels in multi-color imaging, but also identify fluorescent probes that are sensitive to properties of local nanoenvironment at the single-molecule level. By probing compositions, orientations, and electronic structures of individual molecules through their spectroscopic signatures, comprehensive physiological information including, local pH, temperature, viscosity, and protein interaction, may potentially be revealed in cells with nanoscale localization precision. Moreover, the ability to observe and decode the complex behavior of single molecules may also enable quantitative studies of their kinetics, transport, and self-assembly at the fundamental molecular scale. Therefore, sSMLM has the potential to reveal the underlying principles of biological machinery often indistinguishable in conventional experiments. With newly added spectral analysis capability, sSMLM can also be a powerful tool for single-molecule studies to prevent sample misidentification and reduce localization uncertainty. As the existence of fluorescent impurities has been a long-standing obstacle in single-molecule imaging (Wang et al., 2015), the capability of sSMLM allows us to quantify the spatial and spectral characteristics of impurities and effectively separate them from target molecules.

As an emerging imaging technology, the main barrier to advancing sSMLM in multi-color super-

resolution imaging of sophisticated biological processes is the current availability of suitable probes to fully capitalize the newly available spectroscopic analysis capability. Moreover, a comprehensive theoretical framework for examining the trade-offs and evaluating the performances of a sSMLM system has yet to be established. Such a theoretical framework could be particularly valuable, as localization precision and accuracy have been broadly discussed in SMLM and are critical to evaluate the imaging performance. Finally, the development of more compact optical systems and user-friendly data processing tools for sSMLM is necessary for more widespread use of this technique. With further improvement, sSMLM can undoubtedly offer fundamentally new capabilities in many disciplines, from biology to materials science.

Acknowledgment

We acknowledge financial support from National Science Foundation grants CBET-1055379 and EEC-1530734; National Institutes of Health grant R01EY026078; Northwestern University Innovative Initiative Incubator (I3) Award. JLD is supported by National Science Foundation Graduate Research Fellowship Program award 1000231682.

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