Published in final edited form as:

J Phys D Appl Phys. 2019 January 2; 52(1): . doi:10.1088/1361-6463/aae632.

Viewpoint: Localization Microscopy of Single Molecules Enhanced by 3D Imaging and Light Sheet Illumination

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Abstract

Single-molecule super-resolution fluorescence microscopy has produced a revolution in the optical resolution available for study of the nanoscale details of cells and materials. The early work on two-dimensional imaging yielded images of samples that spanned only a small axial range on the order of ~500 nm. Much more useful information can be obtained in complex extended samples using three-dimensional (3D) single-molecule localization. Without 3D capability, visualization of structures extending in the axial direction can easily be missed or confused and the understanding of the science compromised. A variety of methods for obtaining 3D super-resolution images have been devised, each with their own strengths and weaknesses, ranging from point-spread-function engineering to steep axial illumination gradients. Imaging thicker samples, such as mammalian cells and tissue, in all three dimensions presents additional challenges due to increased background and large volumes to image. Light sheet illumination is a method that allows for selective irradiation of the image plane, and its inherent optical sectioning capability allows for imaging of biological samples with reduced background, photobleaching, and photodamage. Beyond these developments, there is a continuing need for better controllable fluorophores, additional novel optical system designs, and enhanced mathematical and statistical signal processing ideas to accurately extract the maximum information available from single emitters in the shortest possible time.

Single-molecule super-resolution (SR) fluorescence microscopy has the powerful ability to illuminate the properties of cells and materials on spatial scales down to tens of nanometers(1, 2). This method generally uses wide-field microscopy and a two-dimensional (2D) camera detector to localize single molecules to much higher precision than the diffraction limit, in a situation where the locations of different single emitting labels are determined at different times by an active control mechanism that limits the concentration of emitters in any one camera frame. Central to this type of imaging beyond the diffraction limit are two key ideas: (a) localization of the single molecule (SM), and (b) control of the concentration of the emitters(3). Localization, the topic of this special issue, involves measurement of the spot from the single emitter (the point-spread function, PSF) and subsequent mathematical estimation of the position of that emitter in the presence of background photons and/or optical aberration, which may be performed by a variety of methods(4) including deconvolution, Fisher information, maximum likelihood estimation,

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and others, including, most recently, deep learning(5). This viewpoint highlights additional recent developments and future directions.

Much more information can be obtained about the system under study by extending from 2D to three-dimensional (3D) single-molecule localization, because without this capability, visualization of structures extending in the axial direction can easily be missed or confused and our understanding of the science compromised. A variety of methods for obtaining 3D single-molecule SR images have been devised, each with their own strengths and weaknesses based on considerations ranging from raw performance to simplicity. Raw performance may be compared by assessing how small the error of x, y, z position information can be; more precisely, what microscope modifications can be made which obtain high Fisher information for both axial and transverse localization. These modifications can be classified based on the optical principle they employ: these include *multifocus* methods, where multiple focal planes are imaged simultaneously; *PSF engineering* methods, where the PSF of the microscope is altered to encode axial position in its shape; and *interference*-, *interface*-, or *intensity-sensing* methods, which measure the position of the molecule relative to features in the imaging geometry instead of fitting the shape of the PSF. Various 3D approaches to both SR and single-particle tracking have been recently reviewed(6).

To illustrate one of the growing strategies for 3D position estimation, Figure 1 shows selected examples of PSF designs for 3D localization: astigmatism, Double-Helix, and Tetrapods, where the images of a single emitter are shown for each in panels (a)-(d). The key point is this: it is the *changes* in the shape of the spot on the camera which encode the axial (z) position, and the different cases shown have usable axial range varying from $\sim 0.8 \mu\text{m}$ to $6 \mu\text{m}$. Although an astigmatic response can be produced by a cylindrical lens, more often it is easier to use a fixed or programmable optical phase mask at an external Fourier plane (conjugate to the back focal plane) created by a $4f$ optical processing setup shown in Fig. 1e. Phase patterns for astigmatism, DH- PSF, and 3 mm Tetrapod are shown in Figs. 1f-h, respectively. It is a fascinating demonstration of the wave-particle duality of light that when the electromagnetic field from a single emitter is properly delayed by these phase patterns at the Fourier plane, the light is reformed to produce the real-space images of Figs. 1a-c, and this self-interference occurs even when only one photon at a time is passing through the optical apparatus.

An important consideration when choosing between the different engineered 3D PSFs is the Cramér-Rao lower bound (CRLB), which reports the best (smallest) localization precision which can theoretically be achieved for an unbiased estimator with a given PSF and underlying noise model including background. The behavior of the CRLB for determining x , y , and z is shown in Fig. 1i, along with the CRLB for the standard (open aperture) microscope. While astigmatism can work well over a short range, the more complex engineered PSFs provide best-case precision that is independent of axial position over much longer ranges. At the same time, tradeoffs naturally emerge for the engineered PSFs, in that the size of the image on the camera is larger than the open aperture PSF, but this can be an easy price to pay for useful 3D information. Further, an increase in the effective axial range (e.g. see the Tetrapod PSF) is accompanied by a slightly poorer CRLB $_z$ near the focal plane.

As always, the CRLB is a theoretical bound, and does not necessarily reflect the true localization precision that is obtained in an experiment which can be affected by choice of estimator, uniformity of background, and optical aberrations. More PSF designs have appeared and will continue to appear in order to provide more optimal performance for certain situations, such as for determination of molecular orientation(7).

While engineered PSFs are a powerful tool for performing precise 3D localization, one immediate obvious application is SR imaging of thick specimens. However, since the SM imaging and localizations are widefield, such samples can introduce a substantial increase in background fluorescence haze from additional molecules at other z positions. A solution to this problem growing in popularity for SM localization applications is light sheet microscopy (Fig. 2), where the pumping light is provided by a thin sheet of light entering from the side and illuminating the image plane of the detection optics. This allows for imaging of the entire transverse image plane simultaneously while providing intrinsic optical sectioning from the illumination of only the current slice, improving contrast by reducing background and preventing premature bleaching of fluorophores. The upper half of Fig. 2 illustrates a range of light sheet approaches and optical configurations each with advantages and disadvantages, recently reviewed in Ref. (8, 9).

To illustrate one of the newest examples, the TILT-3D light sheet microscope combines a tilt of the plane of the light sheet with PSF engineering to generate a simple, efficient design that makes use of the long axial range of the PSFs(10). The lower part of Fig. 2 shows the key idea. Even though the sheet is not parallel to the focal plane, this is not a problem because SM emitters above and below the focal plane are easily imaged, and their axial positions are extracted from the shape of the PSF, not from the position of the light sheet. The focal plane position for each slice is separately measured using a very long range PSF such as the Tetrapod.

In the future, many additional developments are needed and expected in order to push super-resolved localization microscopy into new regimes of resolution and utility. One critical area is the design of fluorophores with higher photon yields with less-perturbative labeling techniques. Given that imaging a larger volume increases the number of labels that must be imaged for a full reconstruction, acquisition rates will become increasingly important, and then it will be necessary to ensure high fluorophore activation or switching rates. It will also be necessary to further develop methods for adaptive optics, potentially correcting at a plane within the sample to accommodate specimen-induced aberrations. Another important area of interest is quantitative microscopy, including methods that measure molecule stoichiometries and clustering, and that generate particle averages between many identical particles in the sample. One can expect cryo-electron microscopy to be further married with optical super-resolution imaging. In all cases, comprehensive approaches to defining overall imaging resolution (11) and fair comparison of the many existing software packages (12) will be very useful in guiding the field.

Acknowledgements:

The author gratefully acknowledges the contributions of members of the Moerner Laboratory over the years, especially Alex von Diezmann, Anna-Karin Gustavsson, Petar Petrov, and Yoav Shechtman for figures. This work was supported in part by the National Institute of General Medical Sciences Grant No. R35-GM118067.

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Figure 1.

Point-spread function (PSF) engineering for 3D imaging. (a)-(d) The PSF shapes are shown as a function of z -position of the emitter. (a) Astigmatic, scale bar $\sim 0.5 \mu\text{m}$, axial range $\sim 0.8 \mu\text{m}$. (b) Double-Helix, scale bar $= 2 \mu\text{m}$, axial range $\sim 3 \mu\text{m}$. (c) A $3 \mu\text{m}$ axial range Tetrapod, scale bar $= 1 \mu\text{m}$. (d) $6 \mu\text{m}$ axial range Tetrapod, scale bar $= 2 \mu\text{m}$. (e) Experimental setup for back focal plane modulation-based PSF engineering where a phase mask is placed in the conjugate Fourier plane. (f)-(h) Phase patterns for selected PSFs: (f) Astigmatic. (g) Double-Helix. (h) $3 \mu\text{m}$ axial range Tetrapod. (i) CRLB values for determining x , y , and z vs axial position for several PSFs. The calculation assumes 1000 detected signal photons, a background of 2 photons per 160 nm pixel, and the use of a $100\times$ oil immersion objective with a NA of 1.4. Adapted with permission from Ref. (6) Copyright (2017) American Chemical Society.

Figure 2.

(Upper) Schematics of different designs that can be used for light sheet single-molecule super-resolution imaging with improved contrast. For discussion of acronyms and references, see Ref. (9) HILO: highly inclined and laminated optical sheet; IML-SPIM: individual molecule localization with selective-plane illumination microscopy; RLSM: reflected light sheet microscopy; LSBM: light-sheet Bayesian microscopy; LLS: lattice light-sheet; soSPIM: single-objective SPIM; SO-LSM: single-objective light-sheet microscopy; TILT3D: tilted light sheet microscopy with 3D. PSFs (10). Schematics are not to scale. Reprinted by permission from Ref. (9), copyright (2018) Optical Society of America. (Lower) Scheme of TILT-3D microscopy. The left side illustrates single-molecule imaging with a shorter-range PSF, such as the DH-PSF, in each position of the light sheet (slice). The single-molecule imaging is interleaved with wide-field imaging of a bright fiducial bead of another color (right side). The axial position of this fiducial relative to each

focal plane position is determined using a very long range PSF such as the 6- μm axial range Tetrapod. Scale bars, 3 μm . Reprinted by permission from Ref. (10).

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