The double-helix microscope super-resolves extended biological structures by localizing single blinking molecules in three dimensions with nanoscale precision

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The double-helix point spread function microscope encodes the axial (z) position information of single emitters in wide-field (x,y) images, thus enabling localization in three dimensions (3D) inside extended volumes. We experimentally determine the statistical localization precision σ of this approach using single emitters in a cell under typical background conditions, demonstrating $\sigma < 20$ nm laterally and < 30 nm axially for $N \approx 1180$ photons per localization. Combined with light-induced blinking of single-molecule labels, we present proof-of-concept imaging beyond the optical diffraction limit of microtubule network structures in fixed mammalian cells over a large axial range in three dimensions.

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Far-field fluorescence microscopy is uniquely suitable for observing the rich structure and dynamics inside biological cells. This capability arises from the relatively non-invasive nature of light and the high molecular specificity afforded by a myriad of tagging schemes for the macromolecules of interest.¹ However, far-field conventional optical microscopy is fundamentally limited to a lateral (x,y) resolution of $\sim \lambda/2$ and an axial (z) resolution of $\sim \lambda$ due to the finite numerical aperture of microscope objective lenses and to unavoidable diffraction of light of wavelength λ . The practical implication of the diffraction limit (DL) is that fluorescent emitters must be separated by more than ~ 200 nm in x,y and ~ 450 nm in z to be resolvable in the visible, thus prohibiting optical investigations at macromolecular scales. Recently, the diffraction limit has been overcome by “super-resolution” (SR) methods of two types: (a) approaches based on spatial patterning of the excitation and/or a nonlinear response (e.g., confocal STED (Ref. 2) or structured illumination³) which directly reduce the size of the point spread function (PSF) and (b) single-molecule methods based on switching or photoactivation to maintain a low emitter concentration in time-sequential wide-field imaging (PALM (Ref. 4)/STORM (Ref. 5)/F-PALM (Ref. 6)).

In the latter class of super-resolution techniques,^{7,8} active control over fluorophore emission is used to maintain a low emitter concentration in any imaging frame. In addition to photoactivation, this may be achieved by photo-physical or photo-chemical processes that render fluorophores temporarily non-fluorescent, such as the well-known on-off “blinking” of single fluorescent molecules,⁹ including fluorescent proteins.¹⁰ As different sparse subsets of molecules labeling the structure of interest fluoresce, they are detected individually on a camera, and their positions are measured with high spatial precision¹¹ by fitting a model function to each multi-pixel single-emitter image. For convenience, these SR methods are referred to as single-

molecule active control microscopies (SMACM).¹² The quality of the resultant image is determined by (1) the statistical precision σ of each individual emitter location and (2) sufficient sampling of the interrogated structure.¹³ The localization precision scales approximately inversely with $\sqrt{N}$, where N is the number of photons detected from the single emitter, but the precision can degrade in the presence of background and excessive pixelation.¹¹

SR imaging in three dimensions (3D) is challenged by the standard fluorescence microscope’s PSF, which contains little information about the axial (z) position of a single emitter. In the axial direction, the PSF is highly symmetric about the focal plane and changes very little over hundreds of nanometers near it. Furthermore, because an objective can only produce a spherical cap of a wavefront of light, the PSF is inherently larger axially than laterally.⁷ This may be addressed by using interferometric approaches with two opposing objective lenses,¹⁴ which yield very high localization precision in 3D,^{15–17} however, the instrumental requirements are considerable and the need for high numerical aperture (NA) objective lenses on both sides of the sample may be impractical to implement for some applications. In comparison, single-lens epi-fluorescence wide-field microscopy is widely utilized in biology, and several approaches have been described for 3D high-precision imaging, including astigmatism,^{18,19} multiplane,^{20,21} and the double-helix PSF,²² the method described here.

In the double-helix PSF (DH-PSF) microscope,²² the PSF response features two prominent spots that revolve around their midpoint throughout the depth of field, thus appearing as a double helix axially. Unlike other PSF schemes that only moderately evolve with z position, the DH-PSF microscope exhibits highly uniform nanoscale localization precision along an axial range of $\sim 2 \mu\text{m}$, which is predicted by information-theoretical calculations²³ and has been demonstrated experimentally.²⁴ In contrast, methods based on astigmatism¹⁸ and biplane²¹ improve the axial localization precision over smaller z ranges.²³

The experimental layout of the DH-PSF microscope has been previously described.²² In brief, after standard Köhler

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FIG. 1. Experimental demonstration of 3D statistical localization precision for single fluorescent small molecules in a fixed mammalian cell. (a) Isolated clusters of localizations from repeated imaging of sparse antibody-bound Atto 655 were analyzed. This example shows 3 clusters (red boxes). (b) Distribution of photons detected above background per localization event for the 880 localizations analyzed in (c), with a median of ~ 1180 photons. (c) Set of 880 localizations from 15 clusters (with 26, 25, 31, 27, 95, 101, 77, 54, 94, 49, 31, 32, 152, 43, and 43 independent localizations each [as in (a)]), translated to a common origin given by each cluster's unweighted center of mass). Histograms of localizations are shown for the x, y, z dimensions along with standard deviations determined from Gaussian fits (red).

illumination in an inverted epifluorescence microscope, emission was collected by a $100\times$ 1.4 NA objective lens (Olympus UPlanSApo) and filtered by a dichroic beamsplitter (Semrock, Di01-R635) and bandpass filter (Omega, 3RD650-710). A $4f$ image processing section relays the fluorescence image onto an electron-multiplying charge-coupled camera, and the standard PSF of the microscope is convolved with the DH-PSF using a reflective phase-only spatial light modulator (Boulder Nonlinear Systems, SLM) placed in the Fourier plane. The DH-PSF phase mask was provided by R. Piestun and S. R. P. Pavani at the University of Colorado.²⁵ Single-molecule localizations were extracted from acquired camera frames using custom-written automated template-matching software that fits recognized images of the DH-PSF to double Gaussian functions.²⁶ For both studies presented here, we excited samples with peak intensities of $\sim 10 \text{ kW/cm}^2$ with 641-nm light from a laser source (Coherent Cube).

In a test experiment (Figure 1) under specific embedding conditions,²⁷ a very sparse set of Atto 655 (Atto-tec) fluorophores were present, conjugated to secondary antibodies for tubulin staining in BSC-1 cells. The fluorophores underwent repeated on-off cycles under the high intensity 641-nm read-out light. In this sparsely labeled sample, each fluorophore

was well isolated, thus providing photon-limited single-molecule emitters [Fig. 1(a)] that served as an experimental measurement of localization precision under realistic biological imaging conditions. Single emitters produced 1180 photons on average above a background of ~ 11 photons/pixel during each imaging frame of 50 ms [Fig. 1(b)]. By translating clusters of independent localizations of isolated single molecules to a common origin [Fig. 1(c)], histograms of localizations were constructed along x, y, z dimensions with their standard deviations determined from Gaussian fits [Fig. 1(c)]. This experiment demonstrates that the DH-PSF microscope affords $\sigma < 20$ nm laterally and < 30 nm axially for $N \approx 1200$ photons detected [Figs. 1(b) and 1(c)]. Since the expected $1/\sqrt{N}$ scaling for localization precision was previously experimentally shown²⁴ to hold for nanoscale fluorescent beads in the range $N = 500$ – 5000 , this measurement provides an independent cell-based calibration for x, y , and z localization precisions for single molecules as a function of collected photons N .

We then performed 3D imaging of microtubules (Figure 2) immunolabeled with Alexa Fluor 647 (Invitrogen) under blinking imaging conditions,²⁸ with glucose oxidase/catalase as an oxygen scavenger system and mM concentrations of mercaptoethylamine (MEA) as the aliphatic reducing thiol necessary for efficient entry into the dark state. A key requirement of DH-PSF-based SMACM is that adjacent double-helix images must not overlap to cause loss of information, and Fig. 2(a) illustrates the efficient generation of resolvable subsets of fluorescent emitters frame-by-frame (15 ms exposure). Notably, new molecules continuously appeared well separated throughout the field of view, allowing the full microtubule structure extending over an axial range of $\sim 1 \mu\text{m}$ to be densely sampled within 300 seconds of imaging. No fiduciary correction for stage drift was needed during this rather short acquisition time. Our results [Figs. 2(b)–2(g)] quantitatively demonstrate nanoscale localization precision as well as the ability to resolve closely spaced objects and to extract the expected shape of a model biological structure.

The pitch, or rotation rate, of the DH-PSF throughout the depth of field may be adjusted, allowing a trade-off between localization precision (and thus imaging resolution) in z and the axial range for detection.²⁹ Our choice of π revolution of the two spots during $\Delta z = 2 \mu\text{m}$ is suitable for capturing the z dynamics of many interesting cellular features. In addition, axial stage motion could be used to further expand the accessible Δz . Other related PSF schemes for nano-localization over a large axial range have also recently been reported.³⁰

Since the resolution enhancement for SMACM methods is governed by the number of photons detected, higher resolution is expected as brighter molecular probes become available. Improvements in precision with this method may also be expected from employing more sophisticated fitting algorithms³¹ than the current double Gaussian fit, which is an approximation of the actual experimental DH-PSF shape. In summary, we have experimentally demonstrated useful 3D super-resolution fluorescence imaging with uniformly high localization precision over a large axial range, localizing biological structures throughout a large field of view with high speed.

FIG. 2. 3D nanoscale imaging in fixed mammalian cells. The BSC-1 microtubules were labeled by standard immunofluorescence with secondary antibodies-Alexa Fluor 647. (a) The DH-microscope images (4 typical frames of 15 ms shown) clearly exhibit double helices above background encoding the 3D coordinates of single emitters. Bar: 5 μm. (b) SR reconstruction of microtubules in 3D over a $\sim 14 \times 14 \mu\text{m}$ field of view. Inset: DL epi-fluorescence image. (c) A different field of microtubules, exploring an axial range of $\sim 1 \mu\text{m}$. In 20 000 frames, 108 337 localizations were collected. The central white box highlights a region containing a large change in z . Bars: 2 μm. (d) Distribution of detected photons above background per localization event for (c). (e) $N^{-1/2}$ power laws for the localization precisions in x, y, z vs. detected photons calibrated by Fig. 1, yielding σ of ~ 14 – 15 nm (x, y) and ~ 25 nm (z) for $N \approx 1490$. (f) and (g) Representative histograms of measured transverse lateral (f) and axial (g) widths of straight 1.5 μm-long microtubule segments (*, †, ‡, **) in (c) indicated by white rectangles, with widths ranging from $\sigma = 40$ to 55 nm laterally and $\sigma = 65$ to 85 nm axially. These measured widths are consistent with the 25 nm diameter size of cylindrical microtubules coated with primary and secondary antibodies (~ 10 nm each) and the present effective imaging resolutions of ~ 33 nm and ~ 60 nm full-width at half-maximum (FWHM), respectively, for the lateral and axial directions (Refs. 28 and 32).

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