WindSTORM: Robust online image processing for high-throughput nanoscopy

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High-throughput nanoscopy becomes increasingly important for unraveling complex biological processes from a large heterogeneous cell population at a nanoscale resolution. High-density emitter localization combined with a large field of view and fast imaging frame rate is commonly used to achieve a high imaging throughput, but the image processing speed and the presence of heterogeneous background in the dense emitter scenario remain a bottleneck. Here, we present a simple non-iterative approach, referred to as WindSTORM, to achieve high-speed high-density emitter localization with robust performance for various image characteristics. We demonstrate that WindSTORM improves the computation speed by two orders of magnitude on CPU and three orders of magnitude upon GPU acceleration to realize online image processing, without compromising localization accuracy. Further, WindSTORM is highly robust to maximize the localization accuracy and minimize the image artifacts in the presence of nonuniform background. WindSTORM paves the way for next generation high-throughput nanoscopy.

INTRODUCTION

Super-resolution localization microscopy, such as stochastic optical reconstruction microscopy (STORM) and (fluorescence) photo-activated localization microscopy [(f)PALM] (1–4), has become an important tool to visualize molecular structures at a nanoscale resolution. Despite its superior spatial resolution, it requires precise localization of sparsely excited single molecules from thousands of frames, which substantial compromises temporal resolution and imaging throughput. High-density emitter localization is an effective strategy to improve the throughput by increasing the emitter density at each frame with a reduced number of imaging frames. To precisely localize the overlapping molecules in dense emitter scenarios while maintaining high localization accuracy (5), complex numerical optimization algorithms are required (6–12). As they are computationally intensive, the long image processing time limits their usage to mostly small image size and short temporal sequence. But the heterogeneous nature of cell populations often requires high-throughput super-resolution imaging on a large number of cells (13). Recent advance in scientific Complementary Metal Oxide Semiconductor (sCMOS) camera technology has greatly benefited super-resolution microscopy with a large field of view (14) and fast frame rate (15), and next-generation high-throughput nanoscopy has become feasible. But as high-throughput nanoscopy can routinely generate gigabyte datasets in seconds, real-time image processing becomes a major challenge. The slow speed of current high-density image processing methods can no longer meet the increasing demand for high-throughput analysis of a huge dataset, while those high-speed image processing methods for sparse emitter scenarios fail in accuracy with unacceptable image quality for high-density data (10, 11). Further, in many high-density scenarios, heterogeneous background is present and can induce significant image artifacts and severely reduces localization accuracy (16). Therefore, an online high-density image processing method that is fast and robust to reconstruct high-quality super-resolution image is crucial for next-generation high-throughput nanoscopy.

We present a computationally simple solution for high-density emitter localization, referred to as WindSTORM, to enable online image processing essential for high-throughput nanoscopy which remains robust even for heterogeneous backgrounds. Unlike conventional high-density emitter localization methods that are based on iterative numerical optimization, our WindSTORM uses non-iterative linear deconvolution to decompose overlapping emitters and retrieve their precise locations. Further, WindSTORM incorporates a new background correction method based on statistical analysis of temporal minimum value that can effectively minimize image artifacts and improve localization accuracy. Through numerical simulation and biological experiments, we demonstrate that WindSTORM achieves real-time image processing of high-throughput nanoscopy on a graphics processing unit (GPU) device and maintains high accuracy and fidelity even in the presence of nonuniform background in various biological samples.

RESULTS

WindSTORM

The central task of a high-density emitter localization method is to identify and localize overlapping emitters. Our WindSTORM achieves robust and high-speed high-density emitter localization via two major steps: (1) overlapping emitter decomposition by background removal and inverse deconvolution with frequency truncation to identify overlapping emitters, and (2) emitter localization via surrounding emitter deduction and central emitter recovery followed by gradient fitting to precisely localize emitters.

The complexity of the overlapping emitter model is the main hurdle for the development of a simple solution to localize overlapping emitters in the high-density emitter scenarios. All existing high-density emitter localization methods rely on numerical optimization with massive iterations to get an approximate estimation of emitter positions (10, 11). Non-iterative linear deconvolution methods (e.g., inverse filtering) are potentially attractive approaches to decompose overlapping emitters but often suffer from serious limitations: Background noise often introduces significant artifacts, limiting the image fidelity and the ability to accurately decompose the overlapping emitters.

WindSTORM overcomes these limitations and uses a computationally simple non-iterative high-density emitter localization method. As
illustrated in Fig. 1, we first perform background subtraction based on extreme value-based emitter recovery (17) to minimize artifacts in linear deconvolution, followed by non-iterative inverse filtering and frequency truncation to decompose the overlapping emitters (see Supplementary Materials). Note that all truncated spatial frequencies are to remove the noise that is much higher than the cutoff spatial frequency determined by the diffraction-limited resolution of the optical system, as indicated by fig. S1. Then, the overlapping emitters can be easily identified by finding their local maxima. Last, for subsequent emitter localization, we apply the surrounding emitter subtraction (see fig. S2 and Supplementary Materials and Methods) (18) to recover the central emitter within each region of interest, which enables us to use a simple algebraic algorithm of gradient fitting (19, 20) for fast emitter localization. As all steps are based on non-iterative operations, we significantly improve the computational speed of WindSTORM on both central processing unit (CPU) and GPU while maintaining a high accuracy for image reconstruction, even in the presence of non-uniform and high background with weak fluorescent emitters.

Numerical simulations

We first benchmarked the performance of WindSTORM against three conventional approaches—a compressed sensing-based approach (implemented in FALCON) (12), a multi-emitter fitting algorithm [implemented in 3D-DAOSTORM (7, 21)], and a single-emitter fitting algorithm [implemented in ThunderSTORM (22)] using simulated datasets with a wide range of emitter densities (0.1 to 6 emitters/\(\mu\)m\(^2\)) in the presence of uniform background. The point spread function (PSF) of each emitter is modeled with the classical Airy pattern derived from the diffraction theory (17), and the emitter intensity is modeled with a log-normal distribution (12). The PSF used in WindSTORM is approximated as a Gaussian function. The performance of emitter localization is quantified by emitter recall rate, root mean square error (RMSE), and false-positive rate in four scenarios—high signal-to-noise ratio (SNR) (Fig. 2A, bright fluorophores such as fluorescent dyes), low SNR (Fig. 2B), ultralow SNR (Fig. 2C), and an extremely low SNR (fig. S3) (weak fluorophores such as fluorescent proteins). For all four scenarios, WindSTORM shows similar recall rate and localization accuracy to those of FALCON and 3D-DAOSTORM and significantly outperform the single-emitter fitting-based ThunderSTORM. Figure 2D shows the comparison of the computational time of these four methods for various emitter densities on both CPU and GPU. At high-density scenarios (e.g., 5 emitters/\(\mu\)m\(^2\)), WindSTORM is about one order of magnitude faster than ThunderSTORM and 3D-DAOSTORM and two orders of magnitude faster than FALCON on CPU, as well as three orders of magnitude faster than FALCON when implemented on GPU.

Next, we evaluated the performance of WindSTORM in a more complex scenario using simulated datasets with dense emitters (5 emitters/\(\mu\)m\(^2\)) of bright emitters [mean (\(\mu\)) = 5000; SD (\(\sigma\)) = 2000 photons] in the presence of nonuniform and high background (ranging from 100 to 1000 photons per pixel per frame) that also undergoes a temporal decay (50%), as shown in Fig. 3 (A to C). Figure 3 (D to G) compares the ground truth (TRUE) with the reconstructed images by WindSTORM, FALCON, and ThunderSTORM, and Fig. 3H compares their localization errors for the recalled emitters. Overall, the single-emitter fitting method (ThunderSTORM) suffers from significantly lower recall rate and higher localization error than WindSTORM and FALCON. In the regions with low background (the edge of the image), the images reconstructed by both WindSTORM and FALCON best match with the ground truth. However, in the middle of the image where non-uniform background is present (Fig. 3F), there is a significant degradation of localization accuracy of FALCON. Figure 3H shows a larger number of emitters with localization error of >20 nm. Similar results were also seen using simulated datasets of weaker emitters with mean

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Fig. 1. The workflow of high-speed high-density localization method—WindSTORM. In our GPU implementation, all steps (inverse deconvolution and emitter localization) were executed on GPU.
Fig. 2. Comparison of different algorithms for the simulated dataset with various emitter densities and intensities. The quantified (A1 to C1) recall rate, (A2 to C2) RMSE, (A3 to C3) false-positive rates, and speed (D) for four algorithms—WindSTORM, FALCON, DAOSTORM, and ThunderSTORM for various emitter densities using simulated datasets. The heterogeneous emitter intensity is modeled with a log-normal distribution with mean and SD of 5000 and 2000 photons for the high SNR case (A1 to A3), 1000 and 400 photons for the low SNR case (B1 to B3), and 350 and 70 photons for the ultralow SNR case (C1 to C3), respectively. (D) The computation time of image reconstruction by WindSTORM, FALCON, 3D-DAOSTORM, and ThunderSTORM on both CPU and GPU for various emitter densities.

Image size: 128 × 128 pixel
Frame number: 500
CPU: Intel i7-4790 @ 3.6 GHz
GPU: Nvidia GTX1080
photon counts of 1500 and SD of 500 in the presence of nonuniform background (50 to 500 photons per pixel per frame), as shown in fig. S4. WindSTORM shows a better accuracy compared to that of FALCON and ThunderSTORM. These results demonstrate the robustness of WindSTORM to accurately reconstruct the super-resolution image in the presence of nonuniform and high background.

**Experimental dataset of STORM imaging with relatively uniform background**

We then assessed WindSTORM using the experimental dataset of dense microtubules labeled with Alexa Fluor 647 from the open-access reference experimental high-density dataset of tubulins (see fig. S5) (11), as well as the experimental dataset obtained by our custom-built STORM system (Fig. 4). In our experimental dataset, the mean photon count is about 10,000. As shown in Fig. 4 (A to D), despite the highly dense emitters in a single frame (Fig. 4A), the background is relatively low and uniform (Fig. 4B) with small temporal variation (Fig. 4C). The super-resolution images reconstructed by WindSTORM (Fig. 4D) and FALCON (Fig. 4E) show significantly higher image contrast with more recalled emitters (4.6 million by WindSTORM and 6.1 million by FALCON) and higher Fourier ring correlation (FRC) resolution (48 nm by WindSTORM and 67 nm by FALCON) than those (2.6 million recalled emitters and 80 nm FRC resolution) by ThunderSTORM (Fig. 4F). The quantified diameter of the microtubules in the reconstructed
image by WindSTORM is also closer to ground truth (24) than FALCON and ThunderSTORM. The results from ThunderSTORM also confirm that the emitter density used here is a high-density scenario where the single-emitter fitting method suffers from low recall rate and localization accuracy. WindSTORM reconstructs the entire image in 11 min on CPU (single core) and 8.5 s on GPU, which are much faster than the speed of conventional high-density localization algorithm FALCON on CPU and GPU. As WindSTORM executed on GPU is faster than the image acquisition time of 100 s, it realizes online image reconstruction for high-density emitters.

Experimental dataset of PALM imaging with nonuniform background

We also tested another scenario of PALM imaging of microtubules labeled with photoactivatable fluorescent protein mEoS3.2, which presents nonuniform background. The mean photon count is about 1000. As shown in Fig. 5 (A to C), the intensity profile along the dashed line in Fig. 5A shows a highly nonuniform distribution, which also undergoes a significant decay over time (Fig. 5C). Figure 5(D to G) compares the diffraction-limited wide-field image (Fig. 5D) with the corresponding super-resolution images reconstructed by WindSTORM.
Fig. 5. Comparison of different algorithms for experimental dataset with dim emitters and nonuniform background. (A) A single-frame raw image of tubulin labeled with mEoS3.2 in COS-7 cells, which presents nonuniform background. The image size is 512 pixels × 512 pixels, and the pixel size on the sample is 81 nm. (B) The cross-sectional profile of the dashed line (L) shown in (A), which shows a nonuniform background. (C) The temporal profile of intensity at three locations (P1, P2, and P3) in (A). (D to G) The diffraction-limited wide-field image (D) and the corresponding super-resolution images reconstructed by WindSTORM (E), FALCON (F), and ThunderSTORM (G). The number of recalled emitters (locs), the FRC resolution, and computational time on the same computing platform are shown at the top of (E) to (G). The zoomed regions in the green boxes 1 and 2 are shown in (D1) to (G1) and (D2) to (G2), respectively. A total of 600 frames were used for image reconstruction.
image by FALCON (Fig. 5E2) exhibits the similar ripple-like structure in the circled region as those in Fig. 5E1, likely an artifact, which is not present in the images reconstructed by WindSTORM and ThunderSTORM. Although the ground truth is unknown, direct comparison between the diffraction-limited and super-resolution images of the same sample has been a well-accepted approach to identify image artifacts in the super-resolution images (25). Further, WindSTORM takes 79 s to reconstruct the image on CPU and 1.8 s on GPU about two to three orders of magnitude faster than FALCON on CPU and GPU. This result demonstrates that WindSTORM is not only significantly faster than other high-density localization algorithms but also more robust to localize weaker emitters (e.g., fluorescent proteins) in the presence of nonuniform background. Additional experimental data on PALM imaging of other subcellular organelles (vimentin and endoplasmic reticulum) are shown in figs. S6 and S7.

**Experimental dataset of bright emitters with nonuniform and high background**

We further evaluated another challenging scenario of imaging densely packed chromatin structure in a tissue section, which often presents heterogeneous strong background due to tissue autofluorescence and stronger scattering. As shown in the representative single-frame raw image (Fig. 6A) of histone mark acetylated H4 (H4Ac) and the intensity profile (Fig. 6C), there is a strong and nonuniform background. We applied WindSTORM, FALCON, and ThunderSTORM to compare the performance of different algorithms. The super-resolution images reconstructed by WindSTORM, FALCON, and ThunderSTORM are shown in (E) to (G) respectively, where number of recalled emitters and computational time on the same computing platform are shown at the bottom right corner. The zoomed region in the green box is shown in (E1) to (G1), and the FRC resolution is shown at the lower right corner. A total of 2000 frames were used for image reconstruction.
background that also undergoes slow decay over time (Fig. 6D). The super-resolution image reconstructed by WindSTORM shows the distinct clusters formed by H4Ac, which is similar to the previously reported spatially segregated nanoclusters in cultured cells via STORM imaging in the presence of low and uniform background (26). However, the super-resolution images reconstructed by FALCON and ThunderSTORM do not show distinct clusters, but more diffuse structures, likely due to compromised resolution. Superior performance is also reflected in the best FRC resolution in the reconstructed image by WindSTORM. In addition, the images reconstructed by FALCON and ThunderSTORM also exhibit background-induced artifacts that are not present in the wide-field image (the region marked by the green circle). To further confirm the presence of clustered structures of H4Ac in the tissue section, we performed STORM imaging of H4Ac on an ultrathin frozen tissue section, where the background is rather low with sparse emitters. We also observed the similar cluster-like structure in the reconstructed super-resolution image (see fig. S8). Further quantification shows that the average cluster size (27) in the super-resolution image reconstructed by WindSTORM is 57 nm, which is the closest to that in the “ground truth” image (51 nm) compared to that in the images reconstructed by FALCON (80 nm) and ThunderSTORM (69 nm). Overall, WindSTORM takes 110 s to reconstruct the image on CPU and only 6.3 s on GPU, much faster than that of FALCON on CPU and GPU. This result further supports that WindSTORM achieves the speed for online image reconstruction while maintaining robust performance for high-density emitters in the presence of nonuniform and high background.

DISCUSSION
As the next-generation super-resolution microscopy moves toward high throughput, it can routinely generate a huge dataset (tens of gigabytes data per minute), where the image reconstruction would take weeks using conventional high-density localization algorithms. We demonstrate a simple non-iterative high-density emitter localization method—WindSTORM—to achieve high-speed and robust image processing for high-density super-resolution localization microscopy. Compared to conventional high-density emitter localization methods, WindSTORM significantly improves the data processing speed by approximately two to three orders of magnitude and remains robust for heterogeneous background; when executed on GPU, real-time image reconstruction can be realized. Besides the significantly faster speed, WindSTORM also achieves high localization accuracy for a wide range of biological samples and image characteristics, especially for weaker emitters, nonuniform and high background, where conventional high-density localization methods often suffer from poor localization accuracy and significant image artifacts.

There are two precautions that users should take when using WindSTORM. First, as WindSTORM is based on fast Fourier transform, the sampling rate of the optical imaging system needs to be adjusted to exceed the Nyquist rate [SD (σ) of the PSF, σ_{PSF} ≈ 1 pixel]. In our experiments, we tested σ_{PSF} of 1.4 pixels (Fig. S5), 1.5 pixels (Fig. 6), and 1.9 pixels (Fig. 5), and all of these oversampling conditions perform well. An undersampling condition may compromise the performance of WindSTORM. We recommend σ_{PSF} of 1.2 to 2.0 pixels to balance sampling rate and SNR. Second, the background estimation algorithm used in WindSTORM is developed on the basis of shot noise model (for sCMOS camera) for temporally slowly varying background. If the Electron Multiplying Charge Coupled Device (EMCCD) camera is used where the excess noise caused by electron multiplication cannot be ignored, then the background correction model needs to be adjusted to account for excess noise (17). However, for high-throughput nanoscopy, a sCMOS camera is highly preferable due to its large field of view and fast frame rate.

In WindSTORM, the width of the PSF needs to be specified as an input parameter. Although this parameter can be easily measured from the experimental data (28), we also explored the scenarios when there is a significant mismatch in PSF width between that used in the inverse deconvolution (WindSTORM) and the actual dataset. Given the Airy-shaped PSF of the optical system with a width of σ = 1.5 pixels, we tested the performance of WindSTORM for mismatched PSF not only in shape (Gaussian-shaped PSF used in deconvolution) but also in PSF width. As shown in fig. S9(A to E), the mismatched PSF width (±0.2 pixel) results in ~1 to 2% reduction in emitter recall rate and ~3 to 4 nm reduction in localization accuracy. Similar results are also found in the experimental dataset, as shown in fig. S9(F to I). Overall, for mismatched PSF between WindSTORM and the actual dataset, the performance of WindSTORM is slightly affected.

In many biological applications, the mixed scenarios of high-density and sparse emitters can be present within the same sample, so the choice of faster image reconstruction speed with single-emitter algorithms may compromise the image resolution. WindSTORM overcomes this limitation, with superior speed, accuracy, emitter recall rate, and minimized artifacts for various imaging conditions compared to conventional localization algorithms. It also eliminates the need for the “expert” users to select the proper localization algorithms (single emitter versus high density) based on the emitter density in the raw image and reduces the barrier for the widespread use of super-resolution localization microscopy.

MATERIALS AND METHODS
Implementation of WindSTORM in GPU
A GPU device has highly parallel computing architecture that consists of thousands of cores and therefore can process the data in parallel at a much faster speed than a CPU device, which only has several cores. Our WindSTORM, including inverse deconvolution, emitter identification and extraction, and emitter localization steps, was fully implemented in GPU to realize online image processing. The inverse deconvolution (Fourier transform) in step 1 is implemented on the basis of the cuFFT library of CUDA, and emitter localization in step 2 was implemented according to the literatures (29), where every emitter was assigned to one thread for parallel processing. Emitter identification and extraction steps, which were often implemented as serial steps in conventional GPU implementation (29), were also fully parallelized in our implementation to boost the image processing speed. The parallelization of these steps simultaneously eliminated the need for frequent rate-limiting data exchange between CPU and GPU, which can be very time-consuming for a large dataset. Thus, our WindSTORM can be robustly executed in GPU and consumes few resources like CPU and memory on personal computers for high-throughput nanoscopy even with a huge dataset.

Numerical simulation
We simulated a series of image sets with a wide range of emitter densities from 0.1 to 6 emitters/μm² commonly seen in super-resolution localization microscopy. Given that the localization error inevitably increases with emitter density regardless of localization algorithms, we
limited our emitter density to be no higher than 5 to 6 emitters/μm² to ensure a satisfactory spatial resolution of ~50 to 60 nm. Further increase of emitter density will come at the expense of localization accuracy (5). The image size was set to be 128 pixels × 128 pixels with a pixel size of 65 nm to mimic a super-resolution microscopy with 100× objective lens [numerical aperture (NA), 1.49] and sCMOS cameras (pixel size, 6.5 μm), and the emitters were randomly distributed in the image. For each image frame, the fluorescence signal was modeled as a distribution of emitters convolved with an Airy pattern, whose kernel width was set to be 1.5 pixels (~100 nm). Emitter intensity was set to follow a log-normal distribution, with mean (μ) and SD (σ) of 5000 photons and 2000 photons, respectively, to mimic bright fluorophores (e.g., Alexa Fluor 647), μ = 1000 photons and σ = 400 photons to mimic the dimmer fluorescent proteins, μ = 350 photons and σ = 70 photons to mimic the ultralow fluorescent intensity (e.g., mEoS3.2), and μ = 150 photons and σ = 30 photons to mimic the extremely low fluorescent intensity. The background per pixel per frame was set to be 50, 20, 10, and 5 photons for bright, dim, ultralow, and extremely low emitter scenarios, respectively.

To test the performance of these algorithms under high and non-uniform background, we simulated a dataset with an emitter density of 500, with a uniform background (5 photons, see fig. S4). Immediately before imaging, PBS buffer was replaced with imaging buffer composed of 10% (w/v) glucose (Sigma-Aldrich), 0.14 M NaCl, 50 mM Tris-HCl (Sigma-Aldrich), and 0.1% Triton X-100 (Sigma-Aldrich) at 1:300 concentration overnight at 4°C. Then, the cells were washed three times with washing buffer. Alexa Fluor 647 (A20106, Thermo Fisher Scientific)—conjugated donkey anti-rabbit secondary antibody (121165, Jackson ImmunoResearch) in blocking buffer at 1:200 concentration was added, then incubated for 2 hours at room temperature, and protected from light.

For tissue imaging, a 3-μm-thick intestinal mouse tissue section was obtained from formalin-fixed, paraffin-embedded tissue block and placed on a coverslip. The tissue section was first deparaffinized in xylene, rehydrated, and followed by heat-induced antigen retrieval performed in the preheated tris-EDTA buffer solution in a microwave oven. The section was then stained with the primary antibody (polyclonal rabbit anti-α-tubulin primary antibody (ab18251, Abcam) at 1:300 concentration overnight at 4°C. Then, the cells were washed three times with washing buffer. Alexa Fluor 647 (A20106, Thermo Fisher Scientific)—conjugated donkey anti-rabbit secondary antibody (121165, Jackson ImmunoResearch) in blocking buffer at 1:200 concentration was added, then incubated for 2 hours at room temperature, and protected from light.

Optical setup and image acquisition
The experiments were performed on our home-built super-resolution fluorescence microscopy based on an Olympus IX71 inverted microscope as described previously (27). The fluorophore (Alexa Fluor 647) or the fluorescent protein (mEoS3.2) was excited by 642- or 560-nm lasers, respectively (VFL-P-1000-642-OEM3 and VFL-P-200-560-OEM1, MPB Communications). The 405-nm laser (DL405-050, CrystaLaser) was used for activation. The laser intensity was controlled by a neutral density filter (NDC-50C-4-A, Thorlabs). The laser beam was expanded by a 10× beam expander (T81-10X, Newport), adjusted for the appropriate field of view. The laser beam was focused onto the rear pupil of a 100× NA 1.4 oil immersion objective lens (UPLSAPO 100XO, Olympus) by an achromatic lens. A high oblique angle was used to illuminate the sample. The fluorescent light was collected by the objective, passing through the dichroic mirror (ZT488/640pc-UFI1, Chroma used for STORM imaging and ZET405/488/561/640m for PALM imaging) and band-pass emission filters (ZET488/640m, Chroma for STORM imaging and ET610/75m for PALM imaging) and then focused by the tube lens onto a sCMOS camera (pco.edge 4.2, PCO-Tech) with a pixel size of 81 nm. For STORM imaging, we acquired 2000 image frames under a 640-nm laser with a power density of 3 kW/cm² and 405-nm laser at a power density of 2.5 W/cm² for activation; for PALM imaging, we acquired 600 to 1000 image frames under 561-nm laser at a power density of 2 kW/cm² for excitation and 405-nm laser at a power density of 2.5 W/cm² for activation. The exposure time was all set as 50 ms.

Sample preparation for PALM imaging
The mEos3.2-Tubulin-C-18, mEos3.2-Vimentin-C-18, and mEoS3.2-ER-5 were gifts from M. Davidson (Addgene plasmid numbers 57484, 57486, and 57455, respectively). Cells were transfected using 2 μl of transfection medium, 100 μl of Opti-MEM (Gibco), 3 μl of Lipofectamine 2000 (Invitrogen), and 1 μg of plasmid per 3.5 cm MatTek glass-bottom dish. After 24 hours of incubation, cells were fixed with 4% paraformaldehyde in PBS for PALM imaging.

Sample preparation for STORM imaging
Mouse embryo fibroblast, COS-7, and U2OS cells were grown at 37°C with 5% CO₂ and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were plated onto a poly-d-lysine–coated glass-bottom dish (FD3510, World Precision Instruments) at about 50 to 70% confluency and incubated overnight to let the cells attach to the dish.

For immunofluorescence staining, cells were pre-extracted for 30 to 60 s in 0.5% Triton X-100 (Triton) in BRB80 buffer supplemented with 4 mM EGTA and fixed with methanol (~20°C) for 10 min. Then, the fixed samples were washed three times with phosphate-buffered saline (PBS). The blocking buffer (3% bovine serum albumin + 0.1% Triton X-100 in PBS) was added to incubate for 1 hour, followed by incubation with rabbit anti-α-tubulin primary antibody (ab18251, Abcam) at 1:300 concentration overnight at 4°C. Then, the cells were washed three times with washing buffer. Alexa Fluor 647 (A20106, Thermo Fisher Scientific)—conjugated donkey anti-rabbit secondary antibody (121165, Jackson ImmunoResearch) in blocking buffer at 1:200 concentration was added, then incubated for 2 hours at room temperature, and protected from light.

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ThunderSTORM was executed on a Quad-core CPU (Core i7-4790, Intel).

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/4/eaaw0683/DC1

Supplementary Materials and Methods
Fig. S1. The workflow of inverse deconvolution and frequency truncation for log-normal intensity distribution with 5000 and 2000 (mean and SD) and 350 and 70.
Fig. S2. Surrounding emitter deduction to recover the central emitter within the region of interest.
Fig. S3. The performance of different algorithms for the simulated dataset with dim emitters and nonuniform background.
Fig. S4. The performance of different algorithms for the open-access experimental high-density localization dataset.
Fig. S5. The performance of different algorithms for reconstructing super-resolution images from experimental dataset of imaging mEos3.2-labeled vimentin.
Fig. S6. The performance of different algorithms for reconstructing super-resolution images from experimental dataset of imaging mEos3.2-labeled endoplasmic reticulum.
Fig. S7. The performance of different algorithms for reconstructing super-resolution images from experimental dataset of imaging nucleosomes labeled with Alexa Fluor 647.
Fig. S8. The effect of mismatched PSF width (ν) between WindSTORM and the actual dataset using both simulated and experimental dataset.
Fig. S9. The effect of mismatched PSF width (ν) between WindSTORM and the actual dataset using both simulated and experimental dataset.
Fig. S10. Relationship between the temporal minima value to the expected background value.

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