We hope that the code and suggestions provided here will inspire other researchers to take advantage of the benefits of using MLE for analyzing Poisson-distributed data, reducing biases and errors in parametric results from histogram-based analysis.

Note: Supplementary information is available on the Nature Methods website.

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QuickPALM: 3D real-time photoactivation nanoscopy image processing in ImageJ

To the Editor: Although conventional microscopes have a resolution limited by diffraction to about half the wavelength of light, several recent advances have led to microscopy methods that achieve roughly tenfold improvements in resolution. Among them, photoactivated light microscopy (PALM) and stochastic optical resolution microscopy (STORM) have become particularly popular, as they only require relatively simple and affordable modifications to a standard total internal reflection fluorescence (TIRF) microscope[1–3] and have been extended to three-dimensional (3D) super-resolution and multicolor imaging[4,5]. PALM and STORM achieve super-resolution by sequentially imaging sparse subsets of photoswitchable molecules. Positions of individual molecules are computed from individual low-resolution images with subdiffraction accuracy. These positions are then corrected for drifts and subsequently assembled into one or more super-resolution images. Unfortunately, in most current implementations, this reconstruction may take from several hours to days for a single dataset, thus forbidding visual inspection of super-resolution images in real time. Additionally, PALM and STORM software used to date is generally not freely available, strongly limiting the adoption of this otherwise relatively simple microscopy method. Two recent publications independently demonstrated two-dimensional (2D) algorithms for real-time PALM and STORM reconstructions[6,7]. Here we present QuickPALM (**Supplementary Software 1**), a freely available and open-source algorithm as a plugin for the widely used ImageJ (http://rsb.info.nih.gov/ij/) software that combines real-time processing capability with additional important features including 3D reconstruction, drift correction and real-time acquisition control (http://code.google.com/p/quickpalm/).

PALM and STORM reconstruction algorithms usually rely on 'fitting' Gaussian kernels to detected diffraction-limited spots. Although they permit high-accuracy localizations, these iterative methods can require up to several hours of processing time. We have developed a high-speed reconstruction algorithm that uses the classical Högbom 'CLEAN' method[8] for spot finding, followed by a modified center of mass algorithm to compute the spot position and parameters defining spot shape along the hori-

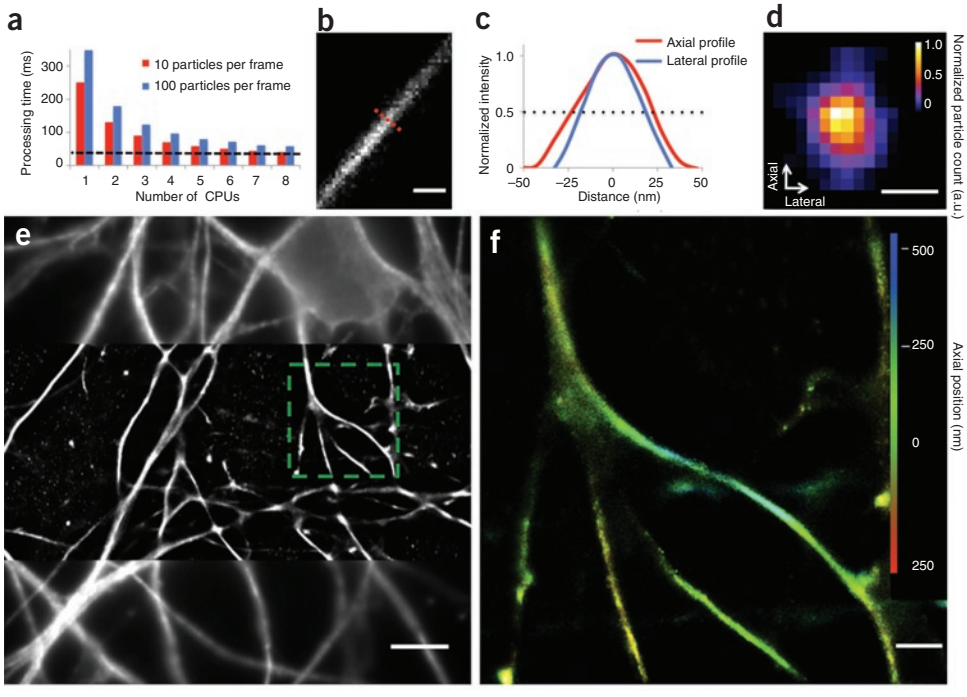

Figure 1 | Three-dimensional dSTORM imaging of β-III-tubulin–labeled microtubules in neuronal cells. (**a**) Performance of the complete algorithm analyzing a sequence of 512 × 512 pixel images including image reading, peak search, localization and super-resolution image rendering in parallel, benchmarked on an Intel Core 2 Quad 3.2 GHz personal computer. The dashed line represents the 32 ms readout speed of the EMCCD camera (Andor iXion-897). Note that this is the lower-bound frame-rate hard limit imposed by the acquisition hardware itself. (**b**) Super-resolution reconstruction of a microtubule filament. (**c**) Intensity profile of the microtubule cross-section (dotted line in **b**) showing a 40 nm lateral and 50 nm axial full-width half maximum. (**d**) Particle distribution histogram of the marked cross-section in **b**. (**e**) The conventional widefield epifluorescence image, with the middle section showing the super-resolved reconstruction of the central portion of the image. (**f**) A 3D dSTORM image of the boxed area in **e**. Scale bars, 100 nm (**b**), 50 nm (**d**), 5 μm (**e**) and 1 μm (**f**).

zontal axes. These parameters allow not only precise 2D localization but also extraction of the axial (z) coordinate for each spot when using an astigmatic lens[5] (**Supplementary Fig. 1** and **Supplementary Note 1**). A direct benefit of this method was our ability to achieve a typical processing time of 30–50 ms per image with a very weak dependence on the number of spots per image (**Fig. 1a**); these values are of the same order as the common electron-multiplying charge-coupled device (EMCCD) maximum frame rates used in these techniques. Any drift on the microscope caused by vibrations or thermal dilation compromises resolution and can lead to artifacts in the reconstruction, such as the doubling of structures for sudden shifts (**Supplementary Fig. 1f,g**). To address this, QuickPALM can trace the movement of fiduciary landmarks such as beads imaged in the sample to estimate the drift in two or three dimensions and subtract it from particle positions (**Supplementary Fig. 1d,e**). As fiduciary landmarks might not be detected throughout the entire image sequence because of blinking or photobleaching, we interpolated missing positions for each landmark (**Supplementary Note 1**).

By combining QuickPALM with the μManager[9] open source package for microscope hardware control and acquisition (**Supplementary Software 1**) in tandem with a custom laser control system (**Supplementary Software 2**), we provide a full method for parallel acquisition and real-time visualization of PALM and STORM experiments.

To test the potential of QuickPALM, we observed microtubules in mouse neurons labeled with a primary antibody to neuronal β-III-tubulin and a secondary Cy5-conjugated antibody (**Fig. 1b–f** and **Supplementary Movie 1**). Using direct STORM (dSTORM) imaging[1,10] we collected 150,000 images, using the 635 nm laser for excitation and pulsing the 488 nm laser at decreasing intervals for Cy5 fluorophore reactivation. We introduced a cylindrical lens at the position of the polarization slider (**Supplementary Methods** and **Supplementary Fig. 2**) to permit 3D probe localization[5]. Using QuickPALM, we obtained real-time 3D reconstructions of microtubules with a resolution (as measured by the full-width half maximum of an intensity profile across a microtubule) of 40 nm in x and y dimensions and 50 nm in the z dimension over a 1 μm depth (**Fig. 1b–f**). With appropriate modifications to ensure precise alignment of differing color channels, multicolor PALM or STORM can be achieved by concurrently processing images from multiple color channels.

In summary, QuickPALM in conjunction with the acquisition control features mentioned above (**Supplementary Discussion**) provides a complete solution for acquisition, reconstruction and visualization of 3D PALM or STORM images, achieving resolutions of ~40 nm in real time. This software package should greatly facilitate the conversion of many laser-excitation widefield or TIRF microscopes into powerful super-resolution microscopes.

Note: Supplementary information is available on the Nature Methods website.

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