

Confined activation and subdiffractional localization enables whole-cell PALM with genetically expressed probes

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We demonstrate three-dimensional (3D) super-resolution microscopy in whole fixed cells using photoactivated localization microscopy (PALM). The use of the bright, genetically expressed fluorescent marker photoactivatable monomeric (m)Cherry (PA-mCherry1) in combination with near diffraction-limited confinement of photoactivation using two-photon illumination and 3D localization methods allowed us to investigate a variety of cellular structures at <50 nm lateral and <100 nm axial resolution. Compared to existing methods, we have substantially reduced excitation and bleaching of unlocalized markers, which allows us to use 3D PALM imaging with high localization density in thick structures. Our 3D localization algorithms, which are based on cross-correlation, do not rely on idealized noise models or specific optical configurations. This allows instrument design to be flexible. By generating appropriate fusion constructs and expressing them in Cos7 cells, we could image invaginations of the nuclear membrane, vimentin fibrils, the mitochondrial network and the endoplasmic reticulum at depths of greater than 8 μm .

The combination of fluorescence microscopy and labeling technologies is an invaluable tool for cell biologists, providing 3D views of protein distributions with high contrast and specificity while minimizing sample perturbation. Despite these advantages, the diffraction limit has placed a lower bound of ~ 250 nm on the smallest structures that can be resolved with optical wavelengths. X-ray microscopy¹ and electron microscopy² provide higher spatial resolution, but usually sacrifice contrast and involve more complex sample preparation.

A number of optical super-resolution techniques can now achieve spatial resolutions down to ~ 20 nm while retaining the advantages of fluorescence microscopy. Structured illumination microscopy³ allows a twofold increase in 3D resolution over the diffraction limit⁴, whereas 3D stimulated emission depletion microscopy⁵ has been demonstrated with an isotropic resolution of ~ 40 nm⁶. A different class of 'pointillist' techniques ((fluorescence) photoactivated localization microscopy, (f)PALM^{7,8}, and

stochastic optical reconstruction microscopy, STORM⁹) rely on repeated stochastic photoactivation of single molecules and their subsequent localization over thousands of wide-field images to provide 20–30-nm resolution in two dimensions⁷ and sub-100-nm resolution in three dimensions¹⁰. Higher resolution can be achieved by combining interferometry with pointillist methods¹¹, but this approach places severe constraints on sample geometry and is limited to a depth within ~ 500 nm of the coverslip.

As pointillist methods build super-resolution images literally molecule by molecule, maximizing the number of successful localizations is crucial for resolving small structures. This procedure depends on isolating the fluorescent signal that is emitted from a single activated molecule from the potentially much larger sea of background arising from cellular autofluorescence and extraneous activation and excitation of other molecules. For surface-bound systems or thin samples (<200 nm), total internal reflection¹² can be used to limit background, allowing even relatively dim, genetically expressed photoactivatable fluorescent proteins (PA-FPs) to be used. These markers are especially useful in super-resolution imaging as they offer greater specificity and effectively higher labeling densities than brighter, exogenously introduced dyes.

Imaging thicker, 3D samples is problematic, as wide-field illumination (Fig. 1a) activates and excites out-of-focus probe molecules, increasing background and generally precluding the use of dim PA-FPs. Furthermore, if an out-of-focus molecule is not localized, it is wasted, decreasing the effective label density and reducing image resolution.

One strategy that alleviates background and is compatible with PA-FPs relies on two-photon illumination to confine activation to the vicinity of the focal plane (Fig. 1b) and subsequent 2D subdiffractional molecular localization under wide-field excitation^{13,14}. As activation is confined to about ± 1 μm of focus, these methods make better use of the available molecular budget than wide-field illumination. However, 2D localization algorithms use only those molecules within ~ 100 nm of focus, wasting the remainder and leaving axial 'gaps' in the reconstructed 3D PALM volume.

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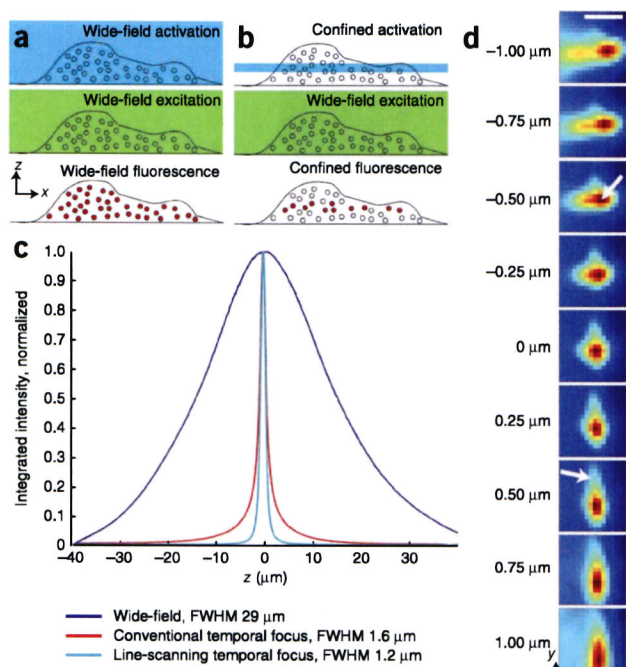


Figure 1 | Experimental considerations for 3D PALM. (a) Wide-field activation (top) and excitation (middle) for localizing molecules (circles) throughout the entire cellular volume (bottom, filled circles). (b) Confined activation with wide-field excitation for localization. (c) Sectioning performance of different illumination modalities. Integrated axial response of ~ 800 -nm-thick quantum dot film, moved through 80- μm axial range in 100-nm steps. Fluorescence was integrated over a $25\ \mu\text{m} \times 25\ \mu\text{m}$ area, yielding the axial response under wide-field (488 nm, blue), conventional temporal focus (800 nm, red) and line-scanning temporal focus (800 nm, cyan) illumination. The last shows reduced FWHM compared to other excitation configurations, and reduced 'tails' at z positions farther from focus. (d) Images of a 100-nm gold bead at different axial positions. The PSF shape is only coarsely approximated by an elliptical Gaussian function, as aberrations cause the intensity center of mass of the PSF to vary axially and 'tails' to vary asymmetrically (arrows). Scale bar, 1 μm .

An alternate strategy for 3D pointillist super-resolution combines wide-field illumination of bright, exogenously introduced dyes with 3D subdiffractional localization techniques¹⁵ for ~ 20 – 30 -nm lateral and ~ 50 – 60 -nm axial localization precision at depths up to 3 μm (ref. 16). By localizing molecules within about ± 300 nm of the focal plane, these techniques better fill the imaging volume, although they still waste localizations and increase background as the entire cell is illuminated simultaneously. The fraction of 'wasted' localizations worsens for thicker samples. This is especially problematic for fluorescent tags that bleach rapidly, such as many PA-FPs.

We combined and improved these two strategies to achieve whole-cell PALM imaging at <100 nm axial and <50 nm lateral resolution with the genetically expressed fluorescent marker photoactivatable mCherry (PA-mCherry1; ref. 17), at depths $>8\ \mu\text{m}$. First, we reduced out-of-focus background by using rapid, line-scanning temporal focusing to confine photoactivation to about ± 600 nm of the focal plane (Fig. 1c). Second, we developed a 3D localization algorithm that works over this full axial range, does not rely on idealized models of optics or noise sources, and is more tolerant of microscope aberrations (Fig. 1d). Compared to existing methods for 3D PALM or STORM, these improvements eliminate axial gaps^{13,14}, mitigate background and reduce wasted probe molecules for improved localization density, thereby enabling super-resolution at greater imaging depths than previously realized. We have demonstrated the utility of this technique by investigating a variety of 3D cellular structures including mitochondria, the endoplasmic reticulum, vimentin filaments and nuclei.

RESULTS

Confined photoactivation for reduced background

PALM and STORM typically use wide-field activation and excitation for sample illumination (Fig. 1a). Although it is easy to implement on epifluorescence microscopes, wide-field illumination

activates and excites molecules outside the focal volume, causing an increased fluorescent background and unnecessary photobleaching. Methods that confine illumination to the focal plane (Fig. 1b), such as two-photon microscopy¹⁸, alleviate these problems.

Conventional temporal focusing¹⁹ microscopy²⁰ confines two-photon illumination to a single plane by scanning an intense line focus across the sample on a picosecond timescale, which allows optical sectioning at depths $>100\ \mu\text{m}$ (ref. 13). This technique can confine the photoactivation of the PA-FP Dronpa²¹ to allow super-resolution imaging in cells at depths up to 5 μm , with ~ 50 nm lateral localization precision¹³. Despite the potential usefulness of the technique, we identified three areas for substantial methodological improvement. First, the best optical sectioning provided by conventional temporal focusing is limited to $\sim 2\ \mu\text{m}$, only half as good as the sectioning of point-scanning two-photon methods. Second, Dronpa is a non-ideal PALM probe with low emitted signal per localization and relatively poor contrast ratio (ratio of fluorescence intensity between activated and nonactivated states). Finally, no attempt was made to combine the technique with axial localization for super-resolution in z .

We improved optical sectioning while maintaining high frame rates by implementing temporal focusing in line-scanning mode²² (Fig. 1c and Supplementary Fig. 1). Line-scanning temporal focusing offers the sectioning quality of point-scanning two-photon microscopy (Supplementary Fig. 2) but the increased scan speed of line-scanning two-photon microscopy by combining spatial focusing and temporal focusing in orthogonal directions. The temporal focus scans an intense point from left to right on a picosecond timescale, illuminating a line, and a galvanometric mirror scans the illuminated line up-down on a millisecond timescale to cover the full imaging field (Supplementary Fig. 3). Acquisition times for conventional point-scanning two-photon microscopy are considerably longer (~ 0.1 – 1 s for the same field of view, a poor match for PALM imaging at high frame rates), although resonant scanning systems are faster (~ 20 ms at the cost of additional instrument complexity) and could be adapted for confined activation. Optimizing line-scanning temporal focusing allowed us to achieve superior axial sectioning with reduced out-of-focus haze and full width half maximum (FWHM; $<1.2\ \mu\text{m}$) compared with conventional temporal focusing and wide-field illumination (Fig. 1c, Online Methods and Supplementary Note 1).

Dronpa has been used in single¹³ and dual-color PALM²³, but emits less fluorescence and has a lower contrast ratio than

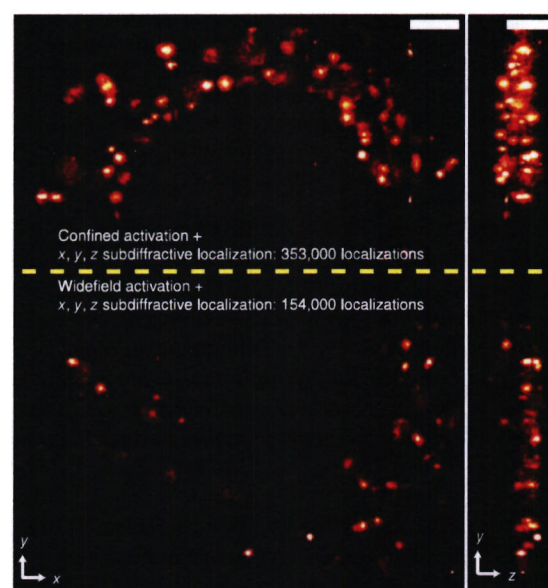
Figure 2 | Improvement over existing 3D PALM. A Cos7 cell expressing PA-mCherry1 targeted to the mitochondrial matrix was simultaneously PALM imaged with both confined (above dashed yellow line) and wide-field (below dashed line) activation. Left: xy maximum-intensity projection. Right: zy maximum-intensity projection. The color map is identical over the entire image. Scale bars, 3 μm .

many PA-FPs, so we searched for alternate PA-FPs that are two-photon activatable. PA-mCherry1¹⁷ is much better suited to PALM, with moderate brightness and high contrast ratio. Upon exposure to an 800-nm activation light, purified PA-mCherry1 proteins on a surface underwent complete photoconversion, and axial activation confinement under scanning temporal focus was improved over conventional temporal focus or one-photon activation (**Supplementary Fig. 4**), prompting us to use this probe for 3D PALM.

3D localization with a measured point spread function

Performing PALM throughout an entire cellular volume with axial resolution significantly below the diffraction limit (~ 750 nm for our 1.2-numerical-aperture (NA) water-immersion objective lens) requires localization in the axial as well as the lateral dimension. Subdiffraction axial localization in the context of 3D whole-cell imaging has been demonstrated using exogenously introduced synthetic dyes^{10,16}, but not with PA-FPs, presumably owing to their lower fluorescence output. Although the nature of the method for subdiffraction localization varies (astigmatism¹⁰, biplane imaging²⁴ or helical point-spread functions (PSFs)²⁵ have been used), an emission PSF that varies rapidly with axial position is common to all methods. After axial variation has been introduced, experimental data are fit to a theoretical model PSF using a least-squares¹⁰ or maximum-likelihood estimator²⁶, which also requires a model of noise. Localization precision therefore depends on how well these models describe experimental conditions, especially when emitted fluorescence is dim, as in the case of PA-FPs. Previous methods used an experimentally measured PSF¹⁵ for 3D super-resolution in biplane PALM²⁴, but such approaches still rely on a model of noise and the measurement of quantities such as total signal photoelectrons and total background photoelectrons.

Experimental noise is difficult to model accurately and background subtraction is complicated in thicker samples where out-of-focus fluorescence varies spatially and temporally during acquisition. Even under constant illumination, different pixels in our charge-coupled device (CCD) chip have different levels of noise and background²⁷. Experimental PSFs are also difficult to model accurately. Introducing a cylindrical lens into our imaging system caused the PSF to resemble an ellipse whose minor and major dimensions varied as a function of z , but close inspection revealed features not easily accounted for by a 2D elliptical Gaussian function as has been used for astigmatism-based localization¹⁰ (**Fig. 1d**; a 2D elliptical Gaussian fit to the molecular image at $z = -1$ μm gives a localization error of hundreds of nanometers). Furthermore, although PSFs were translationally invariant (**Supplementary Fig. 5**), they varied noticeably from experiment to experiment (**Supplementary Fig. 6**; presumably owing to variations in coverslip thickness or tilt²⁸). Rather than attempting to fit a molecular PSF to an *ad hoc* theoretical model function, we directly measured



the 3D PSF of a subdiffraction, nonbleaching gold bead and performed a cross-correlation²⁹ between molecular and bead PSFs to obtain subdiffraction 3D molecular localization (Online Methods and **Supplementary Note 2**), bypassing the problems of a model that imperfectly described our PSF, background and noise. Control experiments comparing the 100-nm gold bead PSF with a ~ 6 -nm quantum dot PSF (closer to the size of PA-FP; **Supplementary Fig. 7**) and comparing images of the gold bead with molecular images at different axial positions (**Supplementary Fig. 8**) showed that our cross-correlation method is viable.

Inherent aberrations of our microscope gave sufficient axial PSF variation to resolve axial steps of 50 nm using a 100-nm gold fiducial bead (**Supplementary Fig. 9**). Adding a cylindrical lens to the beam path improved the signal-to-noise ratio, spreading the signal from out-of-focus molecules over fewer pixels (while introducing a slight but correctable distortion; **Supplementary Fig. 10**), and allowed us to resolve the thickness of a layer of purified PA-mCherry1 molecules (dimmer than a typical fiducial marker) to <150 nm (**Supplementary Fig. 11**). This measurement represents an upper bound on axial localization precision, as coverslip inhomogeneities, surface roughness and tilt broaden the measured thickness of the protein layer, but were not accounted for. The precision of lateral localization is difficult to measure for single PA-mCherry1 molecules, but is ~ 20 – 40 nm for gold fiducial particles of similar brightness (**Supplementary Fig. 12**).

Our algorithm works with different imaging configurations (for example, with or without a cylindrical lens) and is implemented in a free, platform-independent language³⁰ (**Supplementary Software** and <http://code.google.com/p/palm3d/>), allowing this localization method to be performed using any wide-field microscope with a precise axial positioning stage. A 1.4-GHz laptop computer processed ~ 20 – 60 localizations per second per CPU, a total processing time of less than a day for the 3D datasets described below. Images are rendered as histograms (**Supplementary Note 3** and **Supplementary Figs. 13** and **14**), with resolution determined by two factors: localization precision

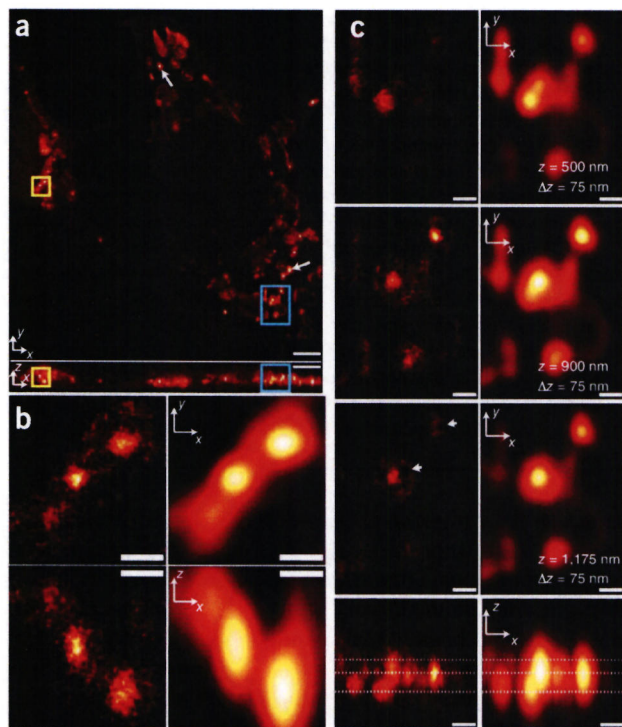


Figure 3 | 3D super-resolution imaging of a mitochondrial network. (a) xy (top) and xz (bottom) maximum-intensity projections of mitochondrial matrix labeled with PA-mCherry1. White arrows show examples of 'core' regions of higher intensity inside mitochondria. About 1.2 million unlinked localizations are rendered in each view. (b) Higher-magnification xy (top) and xz (bottom) views of the yellow rectangular regions in a, with super-resolved (left) and diffraction-limited (right) views compared. (c) Higher-magnification xy sections (top three rows) and xz maximum-intensity projections (bottom row) of the blue rectangular regions highlighted in a. xy sections were constructed from all localizations in a 75-nm-thick z region, and relative z locations are indicated by dotted lines in the xz maximum-intensity projection. Super-resolved (left) and diffraction-limited (right) views are shown. Arrowheads indicate void regions that are invisible in the diffraction-limited views. Only localizations with correlation strength >0.4 are shown. Histogram bin sizes are 60 nm for a and 25 nm for b and c. **Supplementary Video 1** steps through xy slices with 60-nm pixel size and 60-nm z separation. Scale bars, 3 μm (a), 0.5 μm (b,c).

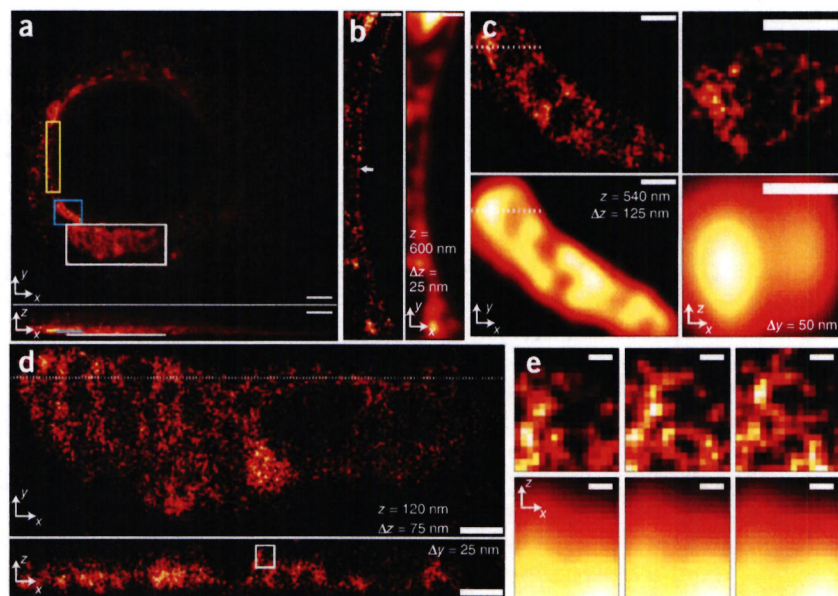
(how precisely the x, y and z coordinates of each molecule are determined) and localization density. Typically our resolution is more limited by localization density than localization precision.

Confined activation with 3D localization improves PALM

To show that the combination of line-scanning temporal focus with our model-independent subdiffraction 3D localization algorithm improves the number of captured localizations compared

with previous wide-field methods¹⁰, we PALM-imaged different regions of a Cos7 cell transfected with PA-mCherry1 fused to the mitochondrial targeting signal of human cytochrome C oxidase subunit VIII with either confined or wide-field illumination (Fig. 2), proceeding until all PA-mCherry1 molecules bleached. By appropriately blocking illumination in image planes conjugate to the sample plane, we restricted line-scanning temporal focus to the top portion of the field of view (above dashed yellow line) and wide-field activation to the bottom portion (below dashed yellow line). In both xy and zy views, confining activation to the vicinity of the focal plane with line-scanning temporal focus increased the number of localizations more than twofold over the $\sim 3\text{-}\mu\text{m}$ -thick sample, and it allowed us to observe mitochondria persisting deeper into the cell than with wide-field activation, as fewer molecules outside the imaging plane were photoactivated but not localized. Using wide-field illumination, most localizations were near the coverslip (Fig. 2), probably because we started imaging

Figure 4 | 3D super-resolution imaging of an endoplasmic reticulum network. (a) xy (top) and xz (bottom) maximum-intensity projections of PA-mCherry1 targeted to the endoplasmic reticulum. About 820,000 unlinked localizations were rendered. (b) Magnified xy views of yellow rectangular region in a, comparing super-resolved (left) and diffraction-limited (right) views. Localizations within a 25-nm z slice centered 600 nm above the coverslip are shown. Arrow highlights nuclear membrane, resolved to ~ 25 nm. (c) Magnification of blue rectangular region in a. Super-resolved xy (left, 125-nm z slice centered 540 nm above coverslip) and xz (right, corresponding to the dotted line in xy view, 50-nm y slice) views are shown in top row, corresponding to the diffraction-limited images in bottom row. (d) Magnification of white rectangular region in a. Super-resolved xy (top, 75-nm z slice centered 120 nm above coverslip) and xz (bottom, corresponding to the dotted line in xy view, 25-nm y slice) views are shown. (e) Magnification of white rectangular region in d, showing super-resolved (top) and diffraction-limited (bottom) xz views. Three successive xz views are shown, 25 nm thick in y and with 25-nm y spacing between views. Localizations with correlation strength >0.4 are shown. Histogram bin sizes: 60 nm (a) and 25 nm (b–e). **Supplementary Video 2** steps through xy slices with 60-nm pixel size and 60-nm z separation. Scale bars, 3 μm (a), 0.5 μm (b–d), 0.1 μm (e).



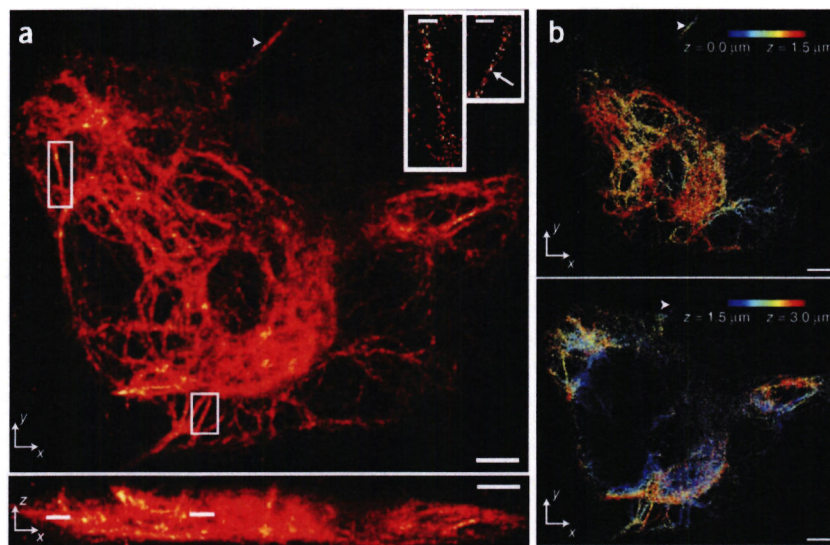


Figure 5 | 3D PALM imaging of a vimentin network. (a) *xy* (top) and *xz* (bottom) maximum-intensity projections of PA-mCherry1-vimentin. About 1 million unlinked localizations were rendered in each view. Insets show further magnification of white rectangles in *xy* (lines in *xz*) maximum-intensity projection, highlighting individual vimentin fibrils in 60-nm-thick *z* slices (localizations are linked). Arrow, region of fibril with apparent width <100 nm. (b) Axial extent of vimentin network with *z* location indicated as a color map. For clarity, localizations corresponding to 0–1.5 μm (top) and 1.5–3 μm (bottom) are shown separately. Arrowheads in **a** and **b** indicate a fibril that persists over >2 μm axially. Only linked localizations with correlation strength >0.4 are shown. Histogram bin sizes are 60 nm for all subfigures. **Supplementary Video 3** steps through *xy* slices with 60-nm pixel size and 60-nm *z* separation. Scale bars, 3 μm (a), 600 nm (insets), 3 μm (b).

there. We obtained similar results in other cells, indicating that confining activation to the vicinity of the focal plane conserves the molecular budget, resulting in more localizations than with wide-field activation. The addition of our 3D subdiffractive localization algorithm substantially improved previous methods that relied on confined activation^{13,14} and 2D subdiffractive localization (**Supplementary Fig. 15**).

3D whole-cell PALM

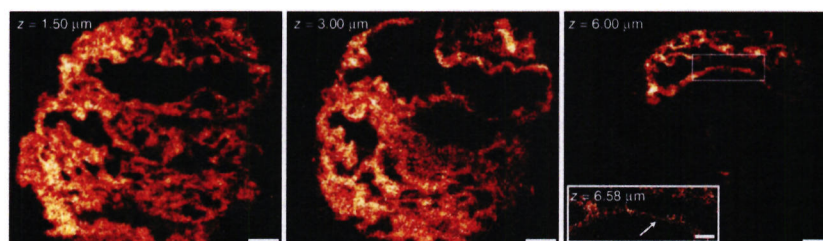
Combining line-scanning temporal focusing for two-photon activation with 3D subdiffractive localization allowed us to ‘fill the gaps’ between 2D imaging planes when PALM-imaging whole fixed cells, and to better conserve the molecular budget available in the sample. We next demonstrated the applicability of the technique by imaging a variety of 3D structures in whole fixed cells (**Figs. 3–6** and **Supplementary Fig. 16**). A 3D PALM histogram of a Cos7 cell transfected with PA-mCherry1 fused to the mitochondrial targeting signal of human cytochrome C oxidase subunit VIII is shown in **Figure 3** and **Supplementary Video 1**. The mitochondrial network spans an imaging field of $\sim 30 \times 30 \mu\text{m}$ over the $\sim 3\text{-}\mu\text{m}$ depth of the cell (**Fig. 3a**). 3D PALM resolved small features such as densely labeled ‘cores’ in mitochondria (whose diameter ranged from <100 nm to >300 nm), surrounded by low-density halos (**Fig. 3a**). A zoomed-in version of three such cores (**Fig. 3a,b**) showed top (*xy*) and front (*xz*) maximum-intensity projections of the core-halo structure, compared to diffraction-limited images (blurred to 250 nm lateral,

750 nm axial resolution). In diffraction-limited images, the core-halo structure is invisible, the axial extent of the cores is distorted, and the leftmost core is nearly obscured. Individual *z*-sections 75 nm thick (**Fig. 3a,c**) reinforce these findings, with several voids (white arrowheads) visible only in the super-resolved images.

As another example of 3D PALM, we PALM-imaged PA-mCherry1 with an N-terminal calreticulin signal peptide and C-terminal KDEL retention sequence, targeting the endoplasmic reticulum (**Fig. 4** and **Supplementary Video 2**). In addition to the membranous honeycomb-like structures previously reported using diffraction-limited fluorescence microscopy³¹, we resolved a thin lining surrounding the nucleus (**Fig. 4a,b**). The apparent thickness of this lining was ~ 25 nm in a 25-nm *z* section (**Fig. 4b**), well below the diffraction limit and suggesting that the endoplasmic reticulum fusion proteins were also targeted at or close to the nuclear membrane. Higher-magnification views of honeycomb endoplasmic reticulum structures are shown in **Figure 4c,d**. Although diffraction-limited imaging resolved large voids in lateral views, internal structure in an *xz* slice was completely obscured (**Fig. 4c**). Fine structure in the higher-magnification view (**Fig. 4d**) was invisible at the diffraction limit, as shown in the higher-magnification *xz* views (**Fig. 4e**). Three successive *xz* slices spaced 25 nm apart in the *y* direction showed that small membrane-enclosed structures persist on smaller length scales, resolved axially to 50–75 nm.

Whole cell 3D PALM can also be applied to filamentous structures, as shown by imaging vimentin–PA-mCherry1 fusions

Figure 6 | 3D PALM image up to $\sim 7.5 \mu\text{m}$ in depth. Fifty-nanometer-thick *xy* slices of PA-mCherry1-lamin B1 fusions in a fixed Cos7 cell, at axial depths of 1.5 (left), 3 (middle) and 6 μm (right); scale bars, 3 μm . A magnified view (inset) of the highlighted region shows a thin section of membrane resolved to ~ 50 nm at an axial depth of 6.58 μm (indicated by arrow); scale bar, 1 μm . Only localizations with a correlation strength >0.4 are shown; bin sizes are 50 nm (main panels) and 25 nm (inset). **Supplementary Video 4** steps through *xy* slices of this 3D image with 50-nm pixel size and 50-nm *z* separation, rendered from ~ 1.9 million unlinked localizations.



(Fig. 5 and Supplementary Video 3). As others have noted in living cells³², we observed a distribution in the size and thickness of vimentin fibrils, with some fibrils appearing as thick as ~650 nm, and others close to our resolution limit (Fig. 5a). The filaments have a complex 3D structure (Fig. 5b; the 3D structure is shown as two 1.5- μm -thick slices to prevent overlapping filaments from obscuring one another). A thick filament extends over >2 μm axially (Fig. 5a,b), confirming that our drift-correction method (Online Methods) aligned images taken at a range of different axial positions.

We resolved subdiffraction nuclear structures at depths up to ~7 μm when imaging PA-mCherry1-lamin B1 fusions (Fig. 6 and Supplementary Video 4). Invaginations of the nuclear envelope were evident, as were void regions of excluded volume. Regions of the nuclear membrane were resolved to <100 nm (Fig. 6). Additional experiments showed that our approach allowed single-molecule imaging at depths >8 μm (Supplementary Video 5).

DISCUSSION

We expect depth-dependent aberrations and scattering to limit our imaging depth, but have not investigated this issue, as our cellular samples were <10 μm thick. Although the methods we developed were motivated to make best use of the limited photon budget of genetically expressed PA-FPs, they might also be advantageous for 3D PALM imaging with other photoactivatable and photoswitchable molecules.

Room for improvement remains. First, acquisition speed might be improved with more laser power (for faster bleaching) and brighter dyes or fluorescent proteins. Given our excitation intensity (~5 kW cm⁻²) and exposure time (100 ms), acquiring data for Figures 3–6 took more than 10 h. Although data processing speed was comparable to acquisition, parallelization with a graphics processing unit (GPU)-based platform²⁶ or cluster would improve computational speed. Second, although we reduced background by limiting photoactivation to the focal plane (requiring a two-photon-activatable dye), we used wide-field excitation. Thus, autofluorescence and spontaneous activation of out-of-focus molecules still impeded single-molecule imaging, especially at greater depths. Further improvement is possible if excitation is also confined to the focal plane, perhaps with selective plane illumination^{33,34} or by using an additional femtosecond laser for excitation as well as activation. Finally, although we consider the generality of our localization algorithm a strength, we suspect accurate models of our PSF and measurement noise would improve localization precision³⁵.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemethods/>.

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

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AUTHOR CONTRIBUTIONS

A.G.Y., A.G. and H.S. conceived, designed and built the experimental setup. A.G.Y. wrote the analysis code. A.G. and H.S. collected the data. A.G.Y., A.G. and H.S. analyzed the data. M.W.D. and A.V. contributed reagents and materials. A.G.Y., M.W.D. and H.S. wrote the paper. All authors edited and refined the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mammalian expression vectors. All PA-mCherry1 (gift from G. Patterson, US National Institutes of Health) expression vectors were constructed using C1 and N1 (Clontech-style) cloning vectors. In all cases, we started with EGFP (enhanced green fluorescent protein) fusion constructs that have been extensively characterized biochemically, cell biologically or both. All DNA for transfection was prepared using the Plasmid Maxi kit (Qiagen). To ensure proper localization, PA-mCherry1 fusion proteins were characterized by transfection in HeLa cells (CCL2 line; ATCC) using Effectene (Qiagen) and ~1 µg vector. Transfected cells were grown on cover glasses in Dulbecco's modified Eagle's medium (DMEM)/F12 medium, fixed after 48 h, and mounted with Gelvatol for imaging. Epifluorescence images (**Supplementary Fig. 16**) were captured with a Nikon 80i microscope coupled to a Hamamatsu ORCA-ER camera system using wide-field illumination and a Texas Red filter set (Omega; QuantaMax Red), showing proper localizations.

The PA-mCherry1 cDNA was amplified with a 5' primer encoding an AgeI site and a 3' primer encoding either a BspEI (C1) or NotI (N1) site for C-terminal and N-terminal fusions (with regards to the fluorescent protein), respectively. The purified and digested PCR products were ligated into similarly digested EGFP-C1 and EGFP-N1 cloning vector backbones. To generate fusion vectors, the appropriate PA-mCherry1 cloning vector and a targeting cDNA PCR amplification product were digested, either sequentially or doubly, with the appropriate enzymes and ligated together after gel purification. Thus, to prepare PA-mCherry1 N-terminal fusions, the N1 cloning vector was digested with NheI and BamHI and treated with calf intestinal alkaline phosphatase (CIAP). cDNA for the mitochondrial targeting signal (MTS) of human cytochrome C oxidase subunit VIII (cDNA source: Origene; NM_004074.2) was amplified to install NheI and BamHI sites containing a 7-amino-acid linker between the targeting DNA sequence and the fluorescent protein sequence. Likewise, cDNA for human vimentin (cDNA source: Origene; NM_003380.3) was amplified to install NheI and BamHI sites containing a 7-amino-acid linker between the targeting DNA sequence and the fluorescent protein sequence. The amplified PCR products were ligated into the pre-cut PA-mCherry N1 cloning vector to yield MTS-7-PA-mCherry-1 (targeting mitochondria) and vimentin-7-PA-mCherry1 (targeting vimentin in the intermediate filament network). To prepare the PA-mCherry1 fusion to lamin B1, the C1 cloning vector was cut with NheI and BglII and treated with CIAP. cDNA for human lamin B1 (DNA source: G. Patterson, National Institutes of Health; NM_005573.2) was amplified to install NheI and BglII sites containing a 10-amino-acid linker between the targeting DNA sequence and the fluorescent protein sequence. The amplified PCR product was ligated into the pre-cut PA-mCherry C1 cloning vector to yield PA-mCherry-1-10-lamin B1 (targeting the nuclear envelope). To prepare the PA-mCherry1 endoplasmic reticulum targeting vector, PA-mCherry1-C1 was cut with BspEI and BamHI and treated with CIAP. A short synthetic oligonucleotide (5'-TCCGGAAAGGACGAGCTGTAAGA ATTC-3') containing a 5' BspEI site and a 3' BamHI site was then ligated into the cloning vector to establish the KDEL sequence at the C terminus of PA-mCherry1 with a 2-amino-acid linker containing a BspEI site. Next, the N-terminal 51 nucleotides of human calreticulin (cDNA source: Origene; NM_004343.3) were amplified using primers encoding a 5' NheI site and a 3' AgeI

site, and this oligonucleotide was ligated into the similarly cut PA-mCherry1-KDEL vector to produce a derivative containing a 5-amino-acid linker between the calreticulin and fluorescent protein sequences and targeting the endoplasmic reticulum. All vector DNA sequences were verified using Big-Dye terminator chemistry on an Applied Biosystems 3130xl Genetic Analyzer with capillary electrophoresis at the Department of Biological Science DNA Sequencing Facility at Florida State University.

Sample preparation. Cos7 cells (ATCC) passages 20–30 were grown in T-25 flasks containing DMEM-HG (Gibco, 31053) supplemented with 10% FBS to 50–70% confluency at 37 °C, 5% CO₂. Trypsinized cells were transiently transfected in 35-mm Petri dishes at 4–7 × 10⁵ cells per dish with Eugene 6 transfection reagent (Roche Applied Science, 11814443001) and 0.5 µg plasmid DNA per dish.

Separately, number 1.5 25-mm diameter glass coverslips (Warner Instruments, CS-25R15) were cleaned as described²³ and coated for ~10 min with 10 µg ml⁻¹ human fibronectin (Millipore, FC010) at room temperature. Transfected cells were then (i) transferred to the coverslips; (ii) grown as above for 36–48 h; (iii) fixed for 15–20 min at room temperature in 4% paraformaldehyde in 1× PBS; and (iv) rinsed three times with PBS containing 5% FBS. Vimentin samples also had 0.1% glutaraldehyde added to the fixation buffer. To compensate for sample drift during imaging, before data acquisition cells were incubated for 15–30 min with 100-nm Au beads (Microspheres-Nanospheres, 790122-010) diluted 1:1 in PBS, before a final rinse with PBS.

Quantum dot samples. Elvax 410 ethylene-vinyl acetate copolymer resin (DuPont) was diluted in toluene to a 4% (w/v) solution by overnight heating at 40 °C. The resulting solution was further diluted to 1% by diluting in a 1:1.5:1.5 4% Elvax 410:Quantum Dots (Q dots; Ocean NanoTech, QSO-520-0010):toluene ratio. The 1% Elvax/Q dot solution was spin coated (Laurell Technologies Corporation, WS-650S-8NPP-LITE) onto a cleaned 25-mm glass coverslip, by ramping from 0 to 500 r.p.m. over 7 s and maintaining 500 r.p.m. for 40 s. Using atomic force microscopy, we verified that the thickness of the spin-coated film was <800 nm.

One-photon laser excitation and activation. The home-built one-photon excitation-activation laser illumination system was based on a described system²³ (**Supplementary Fig. 1**). Two lasers were used: a 200-mW, 561-nm laser (561, CrystaLaser, CL561-200) for excitation and a 100-mW, 405-nm laser (405, Coherent 405-100 CIRCULAR) for activation. Neutral-density filters (ND, Edmund Scientific NT54-460) and half-wave plates (HWP, Thorlabs, AHWP05M-630, WPH05M-405) were used to control the intensity of each beam, and custom beam expanders made from lens pairs were used with each laser (561-nm laser: 2.7× BE, Thorlabs $f = 15$ mm LA1540-A and $f = 40$ mm LA1304-A; 405-nm laser: 2× BE, Thorlabs $f = 15$ mm LA1540-A and $f = 30$ mm LA1289-A) to yield a common beam diameter, thus allowing a common excitation-activation region at the sample.

The 561-nm excitation beam was passed through an acousto-optic tunable filter (AOTF, Quanta Tech, AOTFnc-400.650-TN), used primarily as a fast shutter. The HWPs were used to adjust beam polarization until maximum transmission through the AOTF was achieved. After passing through the AOTF, the 561-nm

beam was combined with the 405-nm activation beam using a dichroic beamsplitter (DC, Semrock, FF458-Di02-25×36).

The co-aligned excitation and activation beams were brought to a focus with one lens (FL) and then re-collimated with another (CL). The entire laser illumination system was positioned relative to the imaging microscope such that the front focal plane of lens CL was coincident with the rear focal plane of a 400-mm achromatic relay lens (RL, Edmund, NT49-369-INK) external to the microscope. By doing so, the focus created by lens FL was imaged at the rear pupil of the microscope objective used for excitation, activation and imaging, resulting in wide-field illumination. The size of the excitation or activation beam could be controlled by varying the focal length of FL or CL; by choosing FL = 50 mm and CL = 100 mm, a spot of approximately ~35- μ m diameter was created at the sample plane.

Two-photon activation, standard temporal focus. A temporal focus module based on a described system¹³ was added to the microscope to confine the activation volume (**Supplementary Fig. 1**). An 80-MHz Ti:sapphire oscillator capable of producing pulses of 140-fs duration and tuned to 800 nm (800, Coherent, Chameleon Ultra II) was used as the two-photon activation source. The 800-nm beam was passed through a Glan-Laser calcite polarizer (POL, Newport, 10GL08AR.16) and an HWP (Newport, 10RP52-2), and the HWP was placed in a motorized rotation mount (Thorlabs, PRM1Z8E) for automated power control. After passing through the polarization optics, the 800-nm beam was passed through a 6.7 $\times$ beam expander (Thorlabs, f = 60 mm AC254-060-B-ML and f = 400 mm AC508-400-B-ML NIR-coated achromats) and directed at 41.6° onto an 830-groove-per-millimeter gold-coated reflective diffraction grating with 21.4° blaze angle (GR, Newport, 53107BK02-035R). The incidence angle of 41.6° was chosen so that the first diffracted order at 800 nm emerged normal to the grating face. The beam was then demagnified 500 $\times$ to produce a field of view of approximately ~35- μ m diameter at the sample plane.

To save space on the optical table, the 500 $\times$ beam demagnification was achieved by placing two expanders in series: an initial de-expansion of 3 $\times$ (Thorlabs, f = 300 mm AC508-300-B-ML and f = 100 mm AC254-100-B-ML) and a secondary de-expansion of 167 $\times$ (Thorlabs, f = 500 mm AC508-500-B-ML and Olympus, UPLSAPO60XW f = 3 mm objective). The total optical path length required from grating to sample was therefore $\sim 2 \times (300 + 100 + 500 + 3) \approx 1.8$ m instead of the $\sim 2 \times (1,500 + 3) \approx 3$ m required if a single f = 1,500 mm lens was used to achieve 500 $\times$ demagnification.

Two-photon activation, line-scanning temporal focus. For improved confinement of activation, we focused the two-photon beam onto a line and scanned the line in the direction perpendicular to the grating grooves, in a setup similar to one described²² (**Supplementary Fig. 1**). A removable mirror mounted on a magnetic base (RM, Thorlabs, KB1X1) was used to divert the 800-nm beam from the standard temporal focus setup described above before beam expansion. The resulting beam was redirected to a galvanometric scanner (GAL, Cambridge Technology, 6215HB), aligned so that the scan direction was perpendicular to the grooves on the diffraction grating. The scan mirror was placed ~400 mm from the diffraction grating GR mentioned above, and an f = 200 mm cylindrical lens (CFL, Thorlabs, LJ1653L2-B) was used to

focus the beam onto the diffraction grating GR. Redirection of the scanned beam onto the grating was achieved with another removable mirror RM. A cylindrical beam expander (CBE, Thorlabs, f = 30 mm, LJ1212L2-B and f = 200 mm, LJ1653L1-B) was placed in the space between galvanometric scanner and grating and was used to expand the beam 6.7 $\times$ in the direction perpendicular to the grating grooves. The combination of the three cylindrical lenses (beam expander plus focusing lens) produced a line focus that could be scanned perpendicular to the grating grooves, in a manner similar to that described²². Subsequent 500 $\times$ demagnification of the line focus was achieved through the beam expanders mentioned above. See **Supplementary Note 1** for further explanation of the optical parameters.

Microscope system. PALM imaging was performed on an Olympus IX81 inverted microscope equipped with differential interference contrast (DIC) optics, an additional side port for introduction of an external laser beam (Olympus, IX2-RFACB2-R), and an automated xy stage with an additional z piezoelectric stage (100- μ m range, Applied Scientific Instrumentation, PZ-2000). The aforementioned 400-mm-focal-length relay lens was used to focus collimated light from the one-photon laser illumination system at the rear pupil of a 60 $\times$, 1.2-NA water-immersion objective (Olympus UPLSAPO60XW). This lens provided good transmission of 800-nm activation light while minimizing spherical aberration and focal shifts due to refractive index mismatch that complicated earlier 3D super-resolution efforts¹⁶. An external dichroic mirror (Semrock, Di01-R561-25×36) placed between the relay lens and the laser port combined one-photon and two-photon illumination sources. Excitation and activation of the sample were achieved by a custom dichroic mirror (Chroma, ZT405/488/561/IR-RPC, reflects 405 nm, 488 nm, 561 nm, 700–1,100 nm) inside a side-mounted Olympus filter cube (Olympus, IX2-MFB-SP-R), and a bandpass emission filter (Semrock, FF01-617/73-25) placed in the same cube served to reject unwanted pump light from the collected fluorescence.

For exciting and imaging PA-mCherry1, the 561-nm laser power was ~85 mW; for one-photon activation experiments, the 405-nm laser power rarely exceeded ~10 μ W; and for two-photon activation, we varied average 800-nm laser power from 0 to 600 mW. All powers were measured immediately before the entrance to the objective. Resulting beam profiles for 561-nm excitation and 800-nm scanned temporal focus activation are shown in **Supplementary Figure 3**. A 1.6 $\times$ expander internal to the microscope was engaged for all PALM measurements, and a 1.2 $\times$ C-mount adaptor (Diagnostic Instruments, DD12BXC) at the microscope output port was used to magnify the final image $60 \times 1.6 \times 1.2 = 115.2\times$ before detection by a back-illuminated, cooled (–65 °C), electron-multiplying CCD camera (EM-CCD, Andor Technology, DU-888E-C00-#BV). The pixel size after magnification was 113 nm. For astigmatic measurements, a 100-mm cylindrical lens (Thorlabs, f = 100mm, LJ1567RM-A) was mounted in a lens tube (Thorlabs, SM1L05) and screwed directly into the female C-mount at the EM-CCD entrance aperture with the appropriate adaptor (Thorlabs, SM1A9). The camera and cylindrical lens system was mounted on a rail system (Thorlabs, RLA0600 and RC1) and translated relative to the microscope output port until the imaging plane was displaced ~1 μ m from the temporal focus plane, as measured by monitoring the signal from 100-nm gold fiducial markers.



Distortion correction. Insertion of the cylindrical lens into the emission path introduced a lateral distortion to the images (**Supplementary Fig. 10**). We characterized the distortion by imaging gold fiducial markers adhered to a glass coverslip and monitoring the displacement in x and y as the microscope stage was moved in defined steps. By noting the apparent position of the fiducial markers versus the stage position, we obtained effective x and y pixel sizes. These pixel sizes (112 nm in x , 147 nm in y) were constant over the imaging field and for a given position of the cylindrical lens. By plotting the pixels as appropriately shaped rectangles, we corrected the images for the imaging distortion introduced by the cylindrical lens.

Drift correction. Mechanical and thermal drift during data acquisition were reduced by a commercial focus lock system (Mad City Labs C-Focus System), mounted directly onto the microscope stage and objective as per the manufacturer's recommendations. We found that the focus lock system reduced axial drift to 200–250 nm over the course of data acquisition. Residual drift is due mostly to thermal or mechanical motion of the coverslip, as the focus lock system is insensitive to motions above the objective. To further correct drift, we monitored the xyz displacement of 100-nm gold fiducial particles (Microspheres-Nanospheres, 790122-010) through their fluorescence (collected through the optics used for monitoring PA-mCherry1 fluorescence), nonspecifically adhered to the coverslip as described above. The combination of fiducial tracking with the focus lock system reduced axial drift to <50 nm.

Data acquisition. We imaged PA-mCherry1 fusions at a frame rate of ~10 Hz, using the highest EM-CCD preamplifier setting of $5\times$ and an EM gain of 300. After a transfected cell was found by exciting the sample at 561 nm, a 'calibration z stack' was taken of nearby fiducial markers. If no fiducials was found near a cell of interest, more fiducials were added until at least one was present in the imaging field. A calibration stack consisted of a series of ten images at each z position, with the z piezo moving from $-2\text{ }\mu\text{m}$ to $+2\text{ }\mu\text{m}$ in steps of 50 nm, and repeated once, for a total of 162 images.

We typically inspected the calibration stack before PALM imaging cells, because although our 3D localization code worked for a range of PSFs, some PSFs were better than others. Strong axial asymmetry of the PSF was often a symptom of spherical aberration, possibly due to variations in coverslip thickness. If the PSF was

excessively dim for positive (or negative) defocus, the signal-to-noise ratio was worse for defocused molecules in that direction. We reduced excessive axial asymmetry by adjusting the correction collar of our objective. Strong bilateral asymmetry of the PSF was typically a symptom of excessive coverslip tilt. This asymmetry did not cause obvious problems for our localization algorithm, unless it opposed the astigmatism of the cylindrical lens in our imaging path. Such antagonistic asymmetry typically gave a PSF that varied slowly in the axial direction, reducing the precision of axial localization. This problem could be mitigated by removing and reinserting the coverslip, taking care to minimize tilt.

After optimizing the PSF, we acquired data at different z piezo positions, separated by 500-nm z steps, often but not always starting at the coverslip surface (for some samples such as PA-mCherry1 lamin constructs, no protein was located at the coverslip surface). We acquired at least 10,000 frames at each z piezo position before advancing 500 nm to the next position, and returned to the same position to acquire more data, until all molecules at a given position were depleted. For z piezo positions other than at the coverslip surface where the fiducial marker was located, we 'jumped' the piezo stage every 200 frames to acquire 20 frames at the z piezo position where the fiducial resided ('fiducial frames'). After each jump, acquisition paused for 30 ms to ensure sufficient 'ring down' time of the piezo stage for stabilization at the new z position. Fiducial frames were combined after acquisition for drift correction.

3D model-independent subdiffractional localization. Following data acquisition, we processed the data in the Python programming language^{30,36}. Our processing code is open-source and freely available (**Supplementary Software** and see <http://code.google.com/p/palm3d/> for the most recent software and documentation). Data processing steps are summarized as follows: (i) construct a calibration stack; (ii) identify candidate particles in each data image; (iii) localize each candidate particle using the calibration stack; (iv) correct for drift; (v) optionally, link localizations and re-localize; (vi) construct image histograms from localization data. Details are available in **Supplementary Note 2**. Image rendering after localization is discussed in **Supplementary Note 3** and **Supplementary Table 1**.

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