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Three-Dimensional Photoactivated Localization Microscopy with Genetically Expressed Probes

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Abstract

Photoactivated localization microscopy (PALM) and related single-molecule imaging techniques enable biological image acquisition at ~20 nm lateral and ~50–100 nm axial resolution. Although such techniques were originally demonstrated on single imaging planes close to the coverslip surface, recent technical developments now enable the 3D imaging of whole fixed cells. We describe methods for converting a 2D PALM into a system capable of acquiring such 3D images, with a particular emphasis on instrumentation that is compatible with choosing relatively dim, genetically expressed photoactivatable fluorescent proteins (PA-FPs) as PALM probes. After reviewing the basics of 2D PALM, we detail astigmatic and multiphoton imaging approaches well suited to working with PA-FPs. We also discuss the use of open-source localization software appropriate for 3D PALM.

Keywords

Super resolution; Single-molecule imaging; 3D microscopy; Cell biology

1 Introduction

Modern fluorescence microscopy is an invaluable tool for the biologist, combining contrast, molecular specificity, and biocompatibility to enable the visualization of cellular constituents. Unfortunately, diffraction limits the spatial resolution of a wide-field fluorescence microscope to ~250 nm laterally and 500–750 nm axially, and achieving even this “diffraction-limited” performance is difficult in practice. Methods for imaging beyond the diffraction limit are thus of great practical interest in cell biology, and the last decade has seen the emergence of such “super-resolution” optical imaging techniques [1].

In this chapter, we focus on photoactivated localization microscopy (PALM) [2] and related single-molecule imaging techniques (e.g., STORM [3], fPALM [4]). In these techniques, the specimen is labeled with a photoswitchable fluorescent probe. During data acquisition, the fluorescence emitted from a sparse subset (“sparse” means that the image of each molecule is resolvable) of probe molecules is isolated by switching on only a few molecules at a time. The fluorescence is recorded using a camera, the probe molecules are switched off, and the process repeated until most or all molecules have been photoswitched. The raw data thus collected consist of thousands of individual frames, each containing diffraction-limited fluorescence images of single photoswitchable molecules. The image of each molecule

(point-spread function or PSF) is analyzed to localize its center with high precision, and the resulting localizations are combined to generate a super-resolution (in optimal conditions, resolutions of ~20 nm or better can be achieved) image of the positions of the molecules (Fig. 1). Resolution is ultimately dependent on (a) localization precision (how precisely each probe molecule may be localized) and (b) density of localizations assembled in the final image (*see* Note 1). Localization precision and density are in turn dependent critically on the choice of switchable molecule, and it is this choice that largely determines the success or failure of a single-molecule super-resolution imaging experiment.

Broadly speaking, photoswitchable probes may be divided into two broad categories: those that may be genetically expressed (e.g., photoactivatable fluorescent proteins, PA-FPs [5]) and those synthetic dyes that may be exogenously introduced via antibodies or small-molecule labeling strategies [6]. Each class has its own strengths and weaknesses: PA-FPs are small (a few nm in size), may be targeted with molecular precision and are thus highly specific, and may possess a high (>1,000) contrast ratio between bright and dark states, helpful in isolating a single fluorescent molecule from its neighbors and acquiring high-density (and thus high resolution) images. Unfortunately, PA-FPs are in general dimmer than most synthetic dyes, limiting localization precision and challenging the detection of activated single-molecule signal above cellular autofluorescence and residual faint background emission from the potentially large number of inactive molecules. They are also available only in limited colors, and the brightness and contrast ratio of the green-emitting PA-FPs is generally lower than the red-emitting PA-FPs (although the palette of PA-FPs continues to expand [7] and the performance of green PA-FPs continues to improve [8]). Switchable synthetic dyes can be very bright and are available in many colors [9], significant advantages over PA-FPs. However, the coupling method (e.g., to antibodies) can introduce significant uncertainty in their position, thus defeating the brightness advantage and effectively limiting resolution. Exogenously introduced dyes often label their target less specifically than PA-FPs, leading to an increased level of background in the reconstructed super-resolution image and sometimes introducing uncertainty as to whether localizations are real or spurious. Finally, many exogenous dyes require an additional chemical cocktail (typically reducing agents or oxygen scavengers) and/or high excitation intensities, limiting application in live cells. In this chapter, we focus on instrumentation particularly suited to the somewhat dimmer PA-FPs.

¹Localization precision (how well the center of each PSF can be determined) and the density of available photoswitchable molecules are two factors that determine the resolution of PALM. In the case of negligible background, the error in the PSF center determination is inversely proportional to the square root of the number of collected photons [16]. Thus, a photoactivatable tag that emits 100-fold more photons than another would give a 10-fold increase in localization precision. Although a high number of collected photons are desirable from a localization standpoint, in practice other factors (errors in the localization estimate, drift correction, background due to other fluorescent tags present at high density, or autofluorescence of the sample) limit the localization precision to ~10 nm for the majority of photoactivatable molecules.

The other key determinant of resolution is the density of molecules. According to the Nyquist-Shannon sampling theorem, the sampling interval (mean distance between neighboring localized molecules) must be at least twice as fine as the desired resolution; otherwise, the feature of interest will be undersampled and unresolved. As an example, to achieve 10 nm resolution in two dimensions, molecules must be spaced 5 nm apart in each dimension, for a minimum density of 4×10^4 molecules/ μm^2 or ~2,000 molecules in a diffraction-limited region 250 nm in diameter. Achieving a high density of localizations in practice is dependent not only on the labeling strategy (PA-FPs vs. exogenous photoswitchable dyes) but also on the contrast ratio (ratio in fluorescence intensity between the inactive and active state of the photoswitchable molecule). If the contrast ratio is less than the density of the probe in a given diffraction-limited region, the weak intensity from the inactive molecules will overwhelm the intensity from a single activated molecule, confounding both localization and the interrogation of densely labeled samples.

Initial PALM experiments were conducted in 2D [2, 10] near the coverslip surface, where optical aberrations and scattering are minimized, and the full numerical aperture (NA) of oil objectives may be used to maximize signal collection and aid in the localization precision of dim PA-FPs. An additional advantage of 2D imaging near the coverslip is that total internal reflection (TIR [11]) may be used to limit excitation to the focal plane and further aid in single-molecule detection. As it forms the basis for 3D experiments, we cover 2D PALM instrumentation in Subheading 3.1.

While suited to membrane [12] and focal adhesion [13] studies in live cells, the obvious drawback of TIRF is that the vast majority of the cell is inaccessible. The simplest way around this problem is to move the excitation out of TIRF, thus employing epi- or wide-field imaging to illuminate the entire cell. This imaging modality also creates several problems for 3D super-resolution imaging: (a) in addition to x and y , a molecule's z coordinate must be localized with subdiffractional precision, and the resulting information used in assembling a 3D PALM dataset; (b) axial drift must be measured and/or controlled; and (c) background photoactivation and subsequent fluorescence of probe molecules at axial locations other than the focal (imaging) plane must be minimized, lest their localizations be wasted and the extraneous background signal so created impede localization in the focal plane. We discuss technical solutions to (a–b) in Subheading 3.2 and (c) in Subheading 3.3. We also refer to the reader to [14, 15], as this chapter draws heavily from these sources.

2 Materials

2.1 2D PALM Instrumentation

1. IX-81 microscope frame equipped with TIRF illuminator, left-side port, DIC optics, filter cube turret, and 1.6× internal beam expander (Olympus America, IX2-RFACB2-R).
2. Automated XY stage (Applied Scientific Instrumentation, MS-2000).
3. High numerical aperture (1.49 NA) 60× objective lens, suitable for TIRF (Olympus America, APON 60XOTIRF).
4. Filters in microscope frame, used for PA-FP imaging:
 - a. mEos2/pa-mCherry1 dichroic mirror (Semrock, FF562-Di02-25×36); bandpass.
 - b. mEos2/pa-mCherry1 emission filter (Semrock, FF01-617/73-25).
 - c. Dronpa/PS-CFP2 dichroic mirror (Chroma, T495lp).

- d. Dronpa/PS-CFP2 bandpass emission filter (Chroma, ET525/50).
5. Optical table (Technical Manufacturing Corporation).
6. Overhead shelf system (Newport, ATS-10).
7. Sticky pads to remove dust (Cole Static Control).
8. Temperature/humidity USB monitor (Practical Design Group LLC, THUM).
9. Excitation optics sled, optomechanics, and spacers for raising sled to TIRF port height (Thorlabs, PBH11106, BLP01, PF175, PS1, PS2, PS3, PS4).
10. Lasers for common PA-FPS:
 - a. Excitation for mEos2, pa-mCherry1, 200 mW, 561 nm (Crystalaser, CL561-200).
 - b. Excitation for Dronpa/PS-CFP2, 150 mW, 488 nm (Newport, Cyan scientific laser).
 - c. Photoactivation of PA-FPs, 100 mW, 405 nm (Coherent, 405-100 CIRCULAR).
11. Sled optics:
 - a. 561 nm beam expansion, 2.7 $\times$, Thorlabs f = 15 mm LA1540-A and f = 40 mm LA1304-A.
 - b. 488 nm beam expansion, 4 $\times$, Thorlabs f = 25 mm AC127-025-A-ML and f = 100 mm AC254-100-A-ML.
 - c. 405 nm beam expansion, 2 $\times$, Thorlabs f = 15 mm LA1540-A and f = 30 mm LA1289-A.
 - d. Neutral density filters (Edmund Scientific, NT54-460).

- e. Cage optomechanics for mounting beam expanders and neutral density filters (Thorlabs, CP02T, ER4, ER6).
- f. Shutter, controller, and power supply (Thorlabs, SH05, TSC001, TCH002).
- g. Visible mirrors and holders (Thorlabs, BB1-E02 and KS1).
- h. External dichroic mirror for integrating 488 nm laser (Semrock, FF506-Di02-25×36).
- i. External dichroic mirror for integrating 405 nm laser (Semrock, FF458-Di02-25×36).
- j. Dichroic mirror holders (Thorlabs, KM100C).
- k. Beam steering assembly (Thorlabs, GN05 and CVI/Melles Griot 12.5 mm diameter/5 mm thick fused silica windows).
- l. Focusing lens (Edmund Optics, $f=50$ mm achromatic lens, NT49-356-INK) and holder (Thorlabs, ST1XY-S and SM1ZM).
- m. Collimating lens (Edmund Optics, $f=100$ mm achromatic lens, NT49-360-INK) and holder (Thorlabs, ST1XY-S and SM1ZM).
- n. Translation stage for imaging focused beam at back focal plane of microscope (Newport, 461-X-M and SM-06).

12. Sample holder and stage insert (Applied Scientific Instrumentation, I-3033 and I-3033-25D).
13. 25 mm, #1.5 coverslips (Warner Instruments, CS-25R15).
14. Back-thinned, electron-multiplying CCD (Andor Technology, DU-888E-C00-#BV).
15. 1.2× emission-side beam expander (Diagnostic Instruments, DD12BXC).
16. Acquisition Computer (Advantech, SYS-4U-BTO with 2 TB hard drive, 2.5 GB RAM, Dual 2.13 GHz processors).

2.2 Additional Components for 3D PALM

1. Automated XY translation stage with z piezo (Applied Scientific Instrumentation, PZ-2000).
2. High numerical aperture (1.2 NA) 60× water-immersion objective lens (Olympus America, UPLSAPO60XW).
3. Focus-lock system to minimize z drift (Mad City Labs, C-Focus).
4. Gold fiducial particles, 100 nm (Microspheres-Nanospheres, 790122-010).
5. Data analysis computer (Xi Computer Corp, 2P64 workstation with 500 GB hard drive, 8 GB RAM, 2× AMD Opteron processors 2 GHz, 8 cores).
6. Network-attached storage device (Amazon.com, Netgear ReadyNAS + 2 TB internal hard drives).
7. Cylindrical lenses for astigmatic localization (Thorlabs, $f=100$ mm, LJ1567RM-A; $f=150$ mm, LJ1629RM-A; $f=300$ mm, LJ1558RM-A).
8. Optomechanics for inserting cylindrical lenses between EM-CCD and microscope left-side port:
 - a. Filter wheel (Thorlabs, CFW6).
 - b. Adapter with external SM1 threads and internal C-mount threads (Thorlabs, SM1A10).
 - c. Adapters with (a) external C-mount threads and internal SM1 threads and (b) external

- d. SM1 threads (Thorlabs, SM1A9 and SM1T2).
- e. 1" travel translation stage (Thorlabs, DT25).
- e. Support for 1.2× expander (Thorlabs, RS2P, extra spacers as needed).

2.3 Components for Two-Photon Activation of PA-FPs

1. Laser for two-photon activation (2PA), capable of producing pulses of 140 fs duration and tuned to 800 nm (Coherent, Chameleon Ultra II).
2. Power control:
 - a. Glan-Laser calcite polarizer (Newport, 10GL08AR.16).
 - b. Half-wave plate for infrared wavelengths (Newport, 10RP52-2).
 - c. Motorized rotation mount (Thorlabs, PRM1Z8E).
 - d. Beam dump (Newport, PL15).
3. Temporal focusing optics:
 - a. Periscope system for directing beam onto scanning mirror from above (Thorlabs, KCB1, CP02T, ER4, ER6, PH3, TR2).
 - b. Galvonometric scanner (Cambridge Technology, 6215HB).
 - c. Function generator for driving galvo (Stanford Research Instruments, DS340).
 - d. Focusing cylindrical lens (Thorlabs, LJ1653L2-B).
 - e. Cylindrical beam expander (Thorlabs, $f = 30$ mm,

	LJ1212L2-B, and $f = 200$ mm, LJ1653L1-B).
f.	Low-dispersion silver mirrors, 1" and 2" diameter (Femtolasers, OA022 and OA248).
g.	830-groove-per-millimeter gold-coated reflective diffraction grating with 21.4° blaze angle (Newport, 53107BK02-035R).
h.	Magnetic mount for diffraction grating (Thorlabs, KB3X3).
i.	Lens pairs for demagnifying grating onto sample (Thorlabs, $f = 300$ mm AC508-300-B-ML and $f = 100$ mm AC254-100-B-ML) and (Thorlabs, $f = 500$ mm AC508-500-B-ML and Olympus, UPLSAPO60XW $f = 3$ mm objective).
4.	Coupling optics for combining 800 nm light with visible wave-lengths used in single-photon excitation:
a.	Removable mirror mounted on a magnetic base (Thorlabs, BB1-E02 and KB1X1).
b.	Visible achromatic lens, $f = 400$ mm (Edmund Optics, NT49-369-INK).
c.	External dichroic mirror for combining visible and 800 nm illumination prior to microscope body (Semrock, Di01-R561-25 $\times$ 36).
d.	Periscope unit for raising 800 nm illumination and directing it into the microscope frame (Thorlabs,

- KS2, RS4P, RS6, RS1, RM1C).
- e. Right-side port attachment on microscope frame for illumination (Olympus, IX2-RFACB2-R).
- f. Filter cube suitable for right-side port illumination (Olympus, IX2-MFB-SP-R).
- g. Custom dichroic mirror to be placed inside filter cube (Chroma, ZT405/488/561/IR-RPC, reflects 405 nm, 488 nm, 561 nm, 700–1,100 nm).
- h. Shortpass emission filter, for blocking 800 nm pump light, to be placed inside filter cube (Semrock, FF01-680/SP-25).

5. Diagnostics for measuring quality of temporal focus:
 - a. Quantum dot solution (Q dots; Ocean NanoTech, QSO-520-0010).
 - b. Ethylene vinyl acetate copolymer resin (DuPont, Elvax 410).
 - c. Toluene (Sigma, 244511).
 - d. Glass vial (Fisher Scientific, 03-339-22C).
 - e. Hot plate (Corning, PC-220).
 - f. Spin coater (Laurell Technologies Corporation, WS-650S-8NPP-LITE).

3 Methods

3.1 2D PALM Instrumentation

As instrumentation for 2D PALM has been covered extensively elsewhere [15], here we review major concepts only. Much of the following description also applies to 3D PALM;

exceptions for TIRF are noted when appropriate. Data acquisition and processing are covered in Subheading 3.2.

3.1.1 Basic Considerations—PALM instrumentation should be housed in a clean, dust-free environment to reduce sample contamination and avoid the stray light and scattering caused by dust in the optical path. We place sticky pads outside the room housing the PALM to help reduce dirt brought in by users. While the undesirable effects of thermal drift may be compensated for by post-processing (Subheading 3.2.4), it is advantageous to control the temperature as much as possible to mitigate this problem. We periodically monitor the temperature using a USB device in order to ensure temperature is within specification. To reduce mechanical vibrations, we bolt all optics on a vibrationally isolated air table (*see* Note 2).

3.1.2 System Overview—Although it is possible to build a PALM system entirely from “the ground up” [2], we find it helpful to use a commercial microscope frame (Olympus, IX-81) as the base because the eyepieces, bright-field optics, objective holder, and filter cube turret make it easy to both screen samples and examine them with additional, non-PALM imaging modes (e.g., differential interference contrast (DIC)). Another advantage of buying a commercial frame is that the TIRF illuminator add-on facilitates TIRF by enabling the user to free-space couple high-intensity lasers of the appropriate wavelength into the system, thus minimizing PALM acquisition time. Also, the microscope manufacturer can provide an automated XY stage (e.g., ASI, MS-2000), helpful in scouting for appropriate samples and in marking their positions. With the frame bolted to the optical table, the main additional components are the excitation and activation optics, the optics housed within the microscope frame itself, and the detection optics (Fig. 2a).

3.1.3 Excitation and Photoactivation—As almost all PA-FPs are photoactivated with near-ultraviolet light, we use the commonly available 405 nm laser for PALM (Coherent, 405-100 CIRCULAR). Red PA-FPs (pa-mCherry1 or mEos2) are efficiently excited with a 561 nm laser (Crystallaser, CL561-200); for green PA-FPs (Dronpa or PS-CFP2), we use a 488 nm laser (Newport, Cyan scientific laser). We bolt all lasers to a small optical sled (Thorlabs, PBH11106), beam expand each to a common diameter, and combine their output with dichroic mirrors (Fig. 2b). We introduce beam-steering assemblies in front of each laser to correct for small differences in laser height or lateral position. Cage-mount optomechanics (Thorlabs, CP02T, ER4, ER6) are particularly useful in mounting beam expanders or neutral density filters and assure colinearity of optics along the beam path. Finally, we place a computer-controlled, mechanical shutter (Thorlabs, SH05, TSC001, and TCH002) after the coaligned beams, allowing the user to easily turn off the illumination to the sample.

3.1.4 Achieving TIRF—As discussed elsewhere [15], TIRF is achieved by focusing the coaligned beams at the edge of the back focal plane (BFP) of the objective lens. We focus the laser illumination on a sled with lens FL and recollimate the beam with lens CL. By

²We find an overhead shelf system (e.g., Newport, ATS-10) useful for mounting laser (and other) power supplies in a space-efficient manner near the optical table.

positioning the entire laser system such that the back focal plane of lens CL is coincident with the front focal plane of the 200 mm relay lens internal to the TIRF illuminator (*see* Note 3), the focus created by FL is imaged at the BFP. In this geometry, the image of the focus can be moved at the BFP by translating a mirror placed on translation stage TS. The “U-DP” attachment on the TIRF illuminator is useful for switching between arc lamp and TIRF illumination, but only the 200 mm relay lens within the illuminator is necessary; the entire fiber-coupling assembly can be removed.

3.1.5 Microscope Body and Sample Holder—Dichroics and emission filters suitable for green or red PA-FPs can be placed directly into commercially available filter cubes and placed into the filter turret assembly (*see* Note 4). Most PALM samples are cells that may be cultured directly on glass coverslips. We use 25 mm round coverslips (Warner Scientific) and place them in a round coverslip holder (ASI, I-3033-25D). The holder and coverslip may be placed inside an insert (ASI, I-3033) that can be secured on the automated stage.

3.1.6 Detection Optics and Camera—For optimal single-molecule localization, the pixel size of the detector must properly sample the point-spread function [16]. Given the pixel sizes of most cameras, this usually implies additional magnification besides the magnification provided by the objective lens. With a 60× objective, we use the 1.6× internal magnifier housed within the microscope frame and a 1.2× external lens, thus providing a total magnification of 115.2 between sample and camera.

We currently employ a back-thinned EM-CCD (Andor, DU-888E-C00-#BV, *see* Note 5) with 13 μm pixels, for a final imaging pixel size of 113 nm.

3.2 3D PALM

With relatively few additional components, the microscope described above can be converted to a 3D PALM system capable of sub-100 nm resolution in all three dimensions. Major additional requirements include a water-immersion objective better suited for 3D samples, minimization and compensation of drift during the acquisition period, cylindrical optics for converting a molecule’s axial position into a PSF shape change, and data processing software capable of processing the raw data into a list of 3D coordinates and rendering the coordinates into a PALM image.

3.2.1 Water-Immersion Objective—For 3D samples that are immersed in aqueous buffer and that are more than a few 100 nm thick, water-immersion objectives are better suited for PALM than the oil objectives used in TIRF. This is for several reasons: (a) the refractive index of most cellular samples is between 1.33 and 1.38; thus, an objective with

³The laser sled’s axial position may be adjusted with appropriate “feet” and spacers from Thorlabs (BLP01, PF175, PS1, PS2, PS3, PS4).

⁴We sometimes find it useful to place an additional bandpass filter (or laser-line blocking filter) into the emission path in order to provide more rejection of the excitation light. A particularly useful component for this is an extra analyzer holder (used in DIC) without the polarizer. This part is available from the manufacturer and provides a convenient space for additional 25 mm diameter optics.

⁵An electron-multiplying charge-coupled device (EM-CCD) camera is still the detector of choice for most single-molecule imaging studies, due to its high quantum efficiency, moderate frame rate, and negligible read noise. We note that EM-CCDs may soon be surpassed by cheaper and faster scientific complementary-symmetry metal-oxide-semiconductor detectors, especially as their quantum efficiency improves.

greater NA does not realize its full resolution potential or collection efficiency. (b) Spherical aberration (due to refractive index mismatch between the oil immersion media and the aqueous sample) causes an oil objective's performance to degrade at increasing depths from the cover-slip. The resulting broadening of the PSF (especially axially) spreads the available signal out, decreasing the signal-to-noise ratio (SNR) per pixel in the raw images. This is especially problematic when localizing dim PA-FPs, where signal is precious and a significant decrease lowers the localization precision or renders the molecule undetectable. At the macroscopic level, the "focal shift" introduced by the refractive index mismatch introduces undesirable axial image distortion. Such deleterious effects may be partially compensated for by adding index-matching reagents to the sample (e.g., glycerol) or by scaling the axial localizations by the ratio of refractive indices [17]. We find it simpler to use a water-immersion objective (Olympus America, UPLSAPO60XW), matching the refractive index of our fixed cellular samples (*see* Note 6) and avoiding these effects while simultaneously maintaining a relatively high NA (1.2) (*see* Note 7). This objective also has good infrared transmission, important for activating PA-FPs with 800 nm light (Subheading 3.3).

3.2.2 Piezoelectric z Stage and Axial Drift Reduction—To assemble a "z stack" or collection of imaging planes that span a 3D sample, it is necessary to move the sample relative to the imaging plane. We use an automated XY stage with an additional z piezo upgrade that permits 100 μm axial displacement of the sample (Applied Scientific Instrumentation, PZ-2000). This replaces the 2D stage in Subheading 3.1.

Drift during single-molecule imaging is a serious problem and must be reduced and/or removed before the final images are rendered. Although it is possible to track fiducial markers (Subheading 3.2.6) to remove the drift after acquisition, the drift during the experiment (especially in z) is sometimes so bad that real-time drift cancellation is needed. "Focus-lock" systems [17] that use a reflection (e.g., from an infrared laser beam) from the coverslip/buffer interface to feedback directly to the piezoelectric stage or objective are likely the best approach, as drift is measured at or near the sample plane. Most major microscope manufacturers and several smaller companies provide these drift-canceling systems as "add-ons" to commercially available microscope frames. Unfortunately, the magnitude of the reflection feedback signal diminishes greatly when refractive index mismatches are minimized (as happens when using a water-immersion objective), so we opt instead for a system that corrects objective drift only (Mad City Labs, C-Focus). This feedback system consists of a piezoelectric collar that houses the objective and contains a glass scale grating and a sensing arm containing a laser and detector. If the reading from the grating changes in response to axial drift, the piezoelectric collar shifts the objective to compensate.

⁶We typically fix samples in a 2–4 % paraformaldehyde/1× PBS (or similar buffer) solution for 15 min, rinsing 3× in 1× PBS before imaging in 1× PBS. A complete protocol is available in ref. 15. We note that fixing in this way and imaging with a water-immersion objective obviates the need for Mowiol or other oil-based mounting schemes and minimizes perturbations to the sample.

⁷Silicone oil objectives may provide a higher NA (1.3) while still minimizing refractive index mismatch, but we have not tested their suitability for PALM.

One drawback of the C-Focus is that it requires both the objective and the sample stage to be raised to accommodate the piezoelectric collar. Although the manufacturer includes spacers for this purpose, we find that the additional circumference of the piezoelectric collar precludes easy use of other objectives in the Olympus filter turret. Rotating the focus-lock housing away from the imaging position in the turret causes it to smash into the turret base. Other objectives may be screwed into the piezoelectric collar to inspect the sample at other magnifications, but this process is somewhat tedious. Nevertheless, we find that we can reduce axial drift to <200 nm over a 15-min period (Fig. 3) using this system, long enough for PALM imaging a single plane in a z stack.

3.2.3 Astigmatic Imaging for 3D Subdiffractive Localization—A variety of methods can be used to reshape the PSF of a subdiffractive particle in order to estimate both axial and lateral position. Such techniques include biplane imaging (introducing defocus into the emission path and collecting two images located at offset z planes, thus providing extra shape information that aids in axial localization) [18], generation of a helical PSF (converting axial position into angular position of a “dumbbell” PSF, usually by placing a spatial light modulator or phase mask in the emission path) [19], and astigmatic imaging (using a cylindrical lens to force rays in vertical and horizontal directions to focus at different axial planes, thus generating a PSF whose ellipticity varies with axial position) [20].

Regardless of the specific implementation, a feature common to all of these methods is that the detected signal in each PSF image is spread out over more camera pixels than in the ideal 2D Airy function. This fact has two consequences: (a) the signal-to-noise per pixel is reduced, reducing localization precision relative to 2D PALM, particularly for dim PA-FPs; and (b) extra care must be taken with the photoactivation laser, to ensure that too many single molecules are not simultaneously activated as their images are larger and thus more likely to overlap.

For dim PA-FPs, the first consequence suggests that maximizing detection efficiency is critical in achieving acceptable SNR and 3D localization precision for PA-FPs. As cylindrical or astigmatic imaging introduces only one additional optical element, minimizing light loss in the emission path, and because it is easy to implement, we chose to adopt this method for 3D localization. When deciding on the placement of the cylindrical lens in the emission path, we considered first placing it beneath the filter cube, in the accessible space where the DIC analyzer usually sits. Unfortunately, we found cylindrical lenses with focal length <1 m (conveniently available from Thorlabs) placed in this location introduced far too much astigmatism in the PSF, thus spreading out the available signal too much and rendering such PSFs unusable for single-molecule localization (*see* Note 8). Instead, we place the cylindrical lens directly after the 1.2× expander external to the microscope and immediately before the EM-CCD (Fig. 4). We find it useful to install the cylindrical lens in a filter holder, permitting us to switch between cylindrical lenses of varying focal length and allowing us the option of operating the microscope with minimal or no astigmatic distortion (to maintain 2D PALM/TIRF operation). Smaller focal length cylindrical lenses introduce greater axial

⁸We note that Rocky Mountain Instruments manufactures cylindrical lenses with up to 10 m focal length.

variation of the PSF, but more image distortion (Fig. 5). The correct pixel sizes to restore the image to the correct aspect ratio can be calculated by measuring the apparent shift of gold fiducial markers (*see* Note 9).

In order to maintain the proper distance between sample and camera, given the extra path introduced by the filter wheel and the adapters, we found it necessary to shave the filter wheel down to the proper thickness. This thickness is best determined empirically, by adjusting the camera position until the image of a thin fluorescent layer is in focus (*see* Note 10). In our system, the total distance between the external surface of the camera and the left edge of the expander is 32 mm, but this distance may vary depending on the exact optics used. Fine adjustments of the axial distance between the filter wheel assembly and left microscope side port may be made by mounting the entire assembly on a translation stage (Fig. 4). In this case, we place a mechanical post beneath the final resting position of the 1.2× expander unit, for mechanical stability (*see* Note 11).

3.2.4 Cross-Correlation Code for 3D Subdiffractive Localization—When using the water-immersion objective, we found it very sensitive to tilt of the coverslip (Fig. 6). This feature has been previously documented [21] and usually introduces aberrations into the PSF that make it hard to model theoretically. Naively fitting a 2D elliptical Gaussian function to the molecular images in Fig. 6 can result in a localization error of hundreds of nanometers. We find it difficult to completely remove this aberration by adjusting the tilt of our coverslip holder, and every time we perform an experiment with a different coverslip, the observed PSFs differ slightly (presumably in response to different degrees of sample tilt). Furthermore, most localization code that relies on experimentally fitting a model function to a PSF also attempts to account for background levels and noise. Experimental noise is

⁹In order to construct a histogram of the processed PALM data, the user must know the nanometers per x and y pixel (“nm_per_x/y_pixel” in the palm3d processing code, code.google.com/p/palm3d/). These values are determined by the magnification of the microscope, the camera’s pixel size, and whether and what focal length cylindrical lens is used. An easy way to determine these quantities is by using the “xy_grid.pgm” and “xy_stage.py” programs (<http://code.google.com/p/palm3d/source/browse/#hg%2Ftest>). These programs record images of a single fiducial marker at its initial position and after defined stage movements in x and y . Creating a maximum projection of all the generated images in ImageJ results in a 5-by-5 grid with 3 μ m spacing between the fiducial location at each node in the grid. The number of pixels between each location in each dimension can then be determined, and dividing the 3,000 nm step size by this value will give the “nm_per_x/y_pixel” to be used for that lens. Before using “xy_grid.pgm,” we set the “kinetic series length” in our camera software to 1, so that only one image is taken at each xy position. Using a field of view with only one fiducial is best, since the presence of additional fiducials will result in a confusing maximum projection with multiple 5-by-5 grids overlapping each other.

¹⁰A good alignment sample is essential for checking the overlap of excitation and activation lasers, as well as measuring the thickness of the 2P temporal focus activation plane. We find a particularly good choice is a thin (<1 μ m) layer of quantum dot nanoparticles, spin coated on a glass coverslip. The quantum dots are bright and photostable, fluoresce over a broad spectral region, and last months if prepared properly.

The quantum dots we start with are bare, i.e., do not have a polymer shell encapsulating them (Q dots; Ocean NanoTech, QSO-520-0010). For this reason, they are hydrophobic (shipped in toluene) and must be embedded in a polymer resin before attachment to a glass coverslip. We prepare a dilution of 4 % (weight by volume) solution of Elvax 410 ethylene-vinyl acetate copolymer resin (DuPont) in toluene by overnight heating on a hotplate. Care must be taken to ensure that the polymer does not bubble, by keeping the temperature stable at or below 40 °C.

We next dilute this solution to 1 % by diluting in a 1:1.5:1.5 4 % Elvax 410/quantum dots/toluene ratio. This solution is stable for ~1 month, if stored in a capped glass vial with a good seal (capping is necessary as the toluene is volatile; glass is important as it is inert and will not be dissolved by toluene). The 1 % Elvax/Q dot solution is spin coated onto a cleaned 25 mm diameter glass coverslip. We use model # WS-650S- 8NPP-LITE (Laurell Technologies Corporation) and ramp the speed from 0 to 500 r.p.m. over 7 s then maintain 500 r.p.m. for 40 s. Other spin coaters work too, and the proper settings may require repeated trials to get a thin layer repeatedly. We find it useful to verify the thickness of the layer with atomic force microscopy, as <1 μ m thickness is preferable for measurement of the thickness of the temporal focus layer. Once made, we store the samples in the dark at room temperature.

¹¹When taking data, we drape black fabric around the entire assembly, thus minimizing external light leakage into the camera.

difficult to model accurately and background subtraction is complicated in thick, 3D samples where out-of-focus fluorescence varies spatially and temporally during acquisition.

For these reasons, rather than using a model PSF that imperfectly reflects reality, we prefer to directly measure the PSF with a 100 nm gold fiducial and perform a cross-correlation between the experimentally measured PSF and the raw single-molecule images to localize each molecule's *xyz* position. This functionality is accomplished using our open-source software program, "palm3d.py," which: (a) crops and smoothes the raw measured PSF data into a calibration stack, (b) identifies candidate molecules from the raw PALM data, (c) localizes the candidates via cross-correlation with the calibration stack, (d) links localizations into molecules and then re-localizes linked localizations (this step is optional), and (e) bins localizations into a 3D histogram. More information about "palm3d.py" and a step-by-step user tutorial can be found at: <http://code.google.com/p/palm3d/>. Other useful programs referenced in the text and notes may be obtained at <http://code.google.com/p/palm3d/source/browse/#hg%2Ftest>.

3.2.5 Control of Data Acquisition—The majority of our hardware is controlled manually during data acquisition. However, our drift correction method requires frequent adjustment of the piezoelectric *z* stage, so we automate this step. We initially used MicroManager to simultaneously control our camera and sample positioning stage but found that MicroManager could not operate the Andor EM-CCD at top speed without crashing occasionally. Since whole-cell PALM can take hours, crashes are unacceptable, and we now use the Andor software (Andor SOLIS for Imaging) that comes with the EM-CCD. Andor's software does not crash during acquisition but presents several serious annoyances. First, the Andor software is "deaf" and cannot be controlled by an external program. Next, it saves in a proprietary, undocumented ".sif" format, which inefficiently uses 32-bit floating-point numbers, even though the camera outputs unsigned 16-bit data. Andor's software can convert the ".sif" format to other formats for processing and viewing, but the batch conversion is buggy and frequently crashes. The Andor software has extremely limited scripting capability and cannot write to a USB serial port, so it cannot directly control our axial positioner. However, the Andor scripting language can issue DOS commands, which is its saving grace. We use this functionality to launch scripts written in Python, which lets us overcome the Andor software's annoyances. During acquisition, one such Python script writes data to the USB serial port to move our piezoelectric *z* stage ("piezo.py"). After each acquisition, Andor's software launches another Python script to convert Andor's ".sif" files to raw binary (see Note 12, "process_palm.py"). Finally, after acquiring calibration images, the Andor software launches a Python program to process the calibration images into a smoothed, cropped estimate of the microscope's point-spread function. We save image data directly to our network-attached storage device (Amazon.com, Netgear ReadyNAS), so the

¹²The main functions of "process_palm.py" are: (a) Conversion of the ".sif" images generated by Andor into ".dat" images (a much easier to use format). Once we realized the ".sif" format appears to consist of a header, followed by numerical data stored as floating-point 32-bit numbers, reverse engineering consisted of learning how to read the header to determine where the numerical data would start and stop within the file. Different versions of the Andor software use slightly different headers, however, so use caution if attempting to reuse our conversion code. (b) Taking the user through cropping and then smoothing the calibration fiducial before generating the cross-correlation plot (see Subheading 3.2.6).

operator can process each dataset as soon as it is acquired without interrupting data acquisition.

3.2.6 Sample Data Acquisition Procedure

1. Alignment: It is a good idea to check the alignment of activation and excitation lasers before data acquisition, especially if the temperature fluctuates. We use a quantum dot sample (*see* Note 10) for alignment, as it fluoresces over a broad excitation wavelength range. After alignment, we set a region of interest (ROI) using camera software. We take care to cover only the illuminated area covered by coaligned activation and excitation beams (we typically use a $\sim 40\ \mu\text{m} \times 40\ \mu\text{m}$ area, 350 by 350 pixels).

2. Fiducials: We minimize axial drift during our potentially long acquisition periods by using a commercial focus-lock system (Subheading 3.2.2, Mad City Labs, C-Focus). To correct for residual drift, we add 100 nm gold particles (Microspheres-Nanospheres, 790122-010) as fiducials (bright particles that do not bleach) to the sample and track their *xyz* displacement over data acquisition.

We typically add $\sim 0.2\text{--}1$ mL of $1\times$ fiducials to our samples and then wait 15–20 min for them to settle. This process should be monitored on the microscope as we have found variability in the “stickiness” of different batches of fiducials and in the same batch over time, which could affect the necessary time or volume to be added. Once a typical field of view has about 1–3 fiducials, the sample is washed with $1\times$ PBS to remove fiducials that have not settled.

3. Calibration: Calibration is the final step that must be performed before data acquisition commences. During calibration, a *z* stack (PSF) is taken of a single fiducial, which is later used during data processing to localize each PA-FP. Calibration should be performed for each sample, as the PSF changes slightly each time a new sample is loaded onto the microscope. We take the *z* stack from $-2\ \mu\text{m}$ to $2\ \mu\text{m}$ ($0\ \mu\text{m}$ is the “best focus” position) in 50 nm steps with ten images taken at each *z* position (later averaging these frames to increase SNR), and repeat this process once to check if thermal drift occurred (a sample calibration program, “calibration.pgm,” is available online).

After the *z* stack is taken, the user selects and crops the fiducial out from the entire field of view. This is a crucial step, and the cropped area should be big enough to include the entire fiducial with the fiducial centered in the cropped area. We find it useful to compute the axial cross-correlation function of the fiducial and display it for the user (“process_palm.py”). Large positive areas in the cross-correlation plot indicate the shape change of the fiducial does not depend strongly on *z*. This is a problem because the calibration data is used during data processing to determine the *z* coordinate of each molecule, and if many different *z* positions look similar, it is difficult to perform a precise *z* localization (Fig. 7).

4. Data acquisition: Once an acceptable calibration has been taken, the user may start acquiring data. An ideal imaging field includes (a) 1–3 fiducials (*see* Note 13), (b) photoactivatable molecules (this can be quickly checked by turning the activation laser on and off and observing the effect on the level of blinking molecules), and (c) low background

(i.e., there should only be blinking where your sample is and not on the coverslip itself). After finding a good field of view, the user should focus on the fiducial (if there are multiple fiducials in the field of view, select the best one as the primary fiducial and focus on it each time) and turn on the focus-lock system. The “track_fid.pgm” program (available online) can then be used for data acquisition.

With thick samples, often the fiducial and the imaging plane are at different z positions, resulting in a dim and out-of-focus fiducial at the desired z position. The “track_fid.pgm” program addresses this potential issue via jumping between the imaging plane and plane of best fiducial focus. Two hundred images are taken at the desired plane (slice images), then 20 images are taken at the z position of the fiducial (tracking images), and this process is repeated a total of 50 times. The program treats the fiducial’s z position as “ $z = 0$ ” and allows the user to determine the z position to image at by modifying the “slicePosition = 0 // $\times 100$ nm” line in the code. During each acquisition round, a total of 11,000 frames are taken, divided amongst slice and tracking images. After completing one acquisition round, the entire process can be repeated at either the same plane or a different plane until all the photoactivable molecules have been bleached. Imaging the same plane multiple times can help add additional detail to that plane, while imaging many different planes (especially when using the cylindrical lens) is advantageous for 3D imaging.

3.3 Two-Photon Photoactivation of PA-FPs for 3D PALM

Close to the coverslip surface (within a few μm), the methods in Subheading 3.2 enable 3D super-resolution imaging with <50 nm lateral and <100 nm axial resolution, if bright and high-contrast photoactivable proteins are used and a sufficiently high density of localizations are extracted during PALM. Further from the coverslip and for thicker samples, astigmatic imaging becomes increasingly difficult for PA-FPs. The main reason for this is that the wide-field illumination employed in Subheading 3.2 activates and excites out-of-focus molecules, increasing background and overwhelming the fluorescence from dim PA-FPs. If an out-of-focus molecule is not detected and localized, it is wasted, decreasing the effective label density and reducing image resolution.

Methods that confine photoactivation to the focal plane, such as two-photon microscopy [22], alleviate such out-of-focus background. As PALM images the entire field at once, the ideal implementation of two-photon activation (2PA) would create a plane of activation at the focal plane. Temporal focusing microscopy [23] achieves such a planar illumination geometry by scanning an intense line focus across the sample on a picosecond timescale. We originally implemented 2PA with temporal focusing in conjunction with the green PA-FP Dronpa, confining activation to $\sim \pm 1$ μm of focus and enabling PALM imaging with lateral localization precisions of ~ 50 nm in single planes throughout whole cells. More recently, we implemented an improved, line-scanning version of temporal focusing [24] that further confines activation to $\sim \pm 0.5$ μm of focus [14]. When combined with the better PA-FP pmCherry1, this implementation enables 3D imaging of whole fixed cells at depths up to 8

¹³Sometimes trash on the coverslip can be mistaken as fiducials; it is useful to inspect the putative fiducial for a few minutes to verify it does not bleach before using it.

μm (Fig. 8 and [14]). We describe how to add such functionality to the microscope described in Subheading 3.2 here.

3.3.1 Femtosecond Laser Choice—A multiphoton laser system suitable for line-scanning temporal focus, 2PA of PA-FPs, must satisfy the following requirements: (a) sufficient average power to drive photoactivation, ideally several watts at 800 nm for maximal photoactivation of pa-mCherry1; and (b) ability to generate pulses of 100–200 fs duration, providing enough dispersion for temporal focusing but not so much dispersion that the pulse broadens unacceptably before reaching the sample. We use the Chameleon Ultra II (Coherent) laser as it satisfies both criteria and is additionally a turnkey system that is easy to use. We note that this laser system is quite expensive (~\$150,000). Cheaper alternatives are available that provide similar pulses and repetition rates but are not tunable. Tunability is not essential, as our experience with Dronpa and pa-mCherry1 suggests that wavelengths near 800 nm are suitable for 2PA of these PA-FPs. The optical setup (Fig. 9a) covered in the following description is optimized for the Ultra II; general considerations that may help in the selection of other laser sources are covered in [14] Supplementary Note 1.

3.3.2 Power Control—As the average power delivered by the laser system may be as high as 4 W, we use polarization optics to control power instead of neutral density filters. As the light from the laser is polarized, we place a half-wave plate (Newport, 10RP52-2) in a motorized rotation mount (Thorlabs, PRM1Z8E) and follow it with a polarizer (Newport, 10GL08AR.16) and beam dump (PL15) (Fig. 9b). During the experiment, we control the degree of photoactivation by controlling the rotation of the half-wave plate from our acquisition computer.

3.3.3 Optical System for Achieving Line-Scanning Temporal Focus—Line-scanning temporal focusing offers the sectioning quality of point-scanning two-photon microscopy 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 (by illuminating the diffraction grating with a line focus), and a galvanometric mirror scans this illuminated line up-down on a millisecond timescale to cover the full imaging field. The task for the user thus involves focusing the 2P excitation into a line focus onto the grating, and imaging the surface of the grating onto the sample plane. After directing the beam onto a galva-nometric scanner (Cambridge Technology, 6215HB), we place a 200 mm focal length cylindrical lens (Thorlabs, LJ1653L2-B) 200 mm from the galvo and focus the excitation into a line shape on a diffraction grating (Newport, 53107BK02-035R) placed 200 mm from the cylindrical lens. The galvo is oriented so that the scan direction is parallel to the grooves on the grating; this necessitates a mounting scheme that directs the beam onto the galvo from above. We achieve this by periscoping the beam up and directing it downwards onto the galvo (Fig. 9c). We direct the beam onto the grating face at 41.6° incidence angle (Fig. 9d), chosen so that the first diffracted order at 800 nm emerges normal to the grating face. In the intermediate space between galvo, cylindrical lens, and grating, we place a cylindrical lens beam expander, thus expanding the beam 6.7× in the direction perpendicular to the grating grooves

(this cylindrical lens expander is thus oriented so that it expands the beam in a direction perpendicular to the focusing cylindrical lens).

To achieve near-diffraction-limited sectioning, it is important to demagnify the image of the grating enough so that the back focal plane is overfilled [14]. We achieve this condition by using a demagnification of $500\times$ between grating and sample. Given the 3 mm focal length of our objective lens, a single $f = 1,500$ mm lens after the grating would accomplish this job but would require a total of $\sim 2 \times (1,500 + 3) \approx 3$ m path length on our optical table. A more space-efficient solution is to break up the demagnification with two telescopes: a 3:1 demagnification followed by a $167\times$ demagnification (Fig. 9a).

3.3.4 Injection of 2P Illumination into Microscope and Combination with Visible Illumination—We find it easiest to couple 2P illumination into the optical system by using the Olympus laser port B addition to the filter cube turret. Using this accessory requires: (a) periscoping the 2P illumination beam to the height of laser port B, (b) combination with 1P excitation light from the excitation sled (Fig. 9e), and (c) appropriate optics housed within the microscope body.

We periscope the scanned temporal focus activation beam with the aid of 2" mirrors (Femtolasers, OA022 and OA248) attached to a pillar post (Thorlabs, RS4P, RS6, RS1) with appropriate opto-mechanics (Thorlabs, KS2, RM1C). To couple the visible laser output from the excitation sled into laser port B, while maintaining the possibility of TIRF excitation, we place a removable mirror in front of the TIRF port. With the removable mirror in place, visible light is reflected into laser port B with a dichroic mirror (Semrock, Di01-R561-25 $\times$ 36) designed to reflect wavelengths <561 nm but transmit the 800 nm illumination emerging from the periscope. We place a 400 mm lens (Edmund Optics, NT49-369-INK) in the visible path before the dichroic, thus focusing the visible light to the objective back focal plane and enabling wide-field excitation. Finally, we place a custom dichroic mirror (Chroma, ZT405/488/561/IR-RPC) that reflects 405, 488, 561, and 700–1,100 nm illumination while transmitting the intermediate wavelengths into a side-mounted filter cube (Olympus, IX2-MFB-SP-R) placed in the filter turret. We also place a shortpass emission filter (Semrock, FF01-680/SP-25) into this cube, thus blocking transmitted infrared light from reaching the camera. As this filter does not leave room for the emission filter typically used for PA-mCherry1 (Semrock, FF01-617/73-25), we instead place it in an empty DIC filter holder and place it beneath the filter turret.

3.3.5 Alignment of 2P Excitation Optics and Matching Plane of Temporal Focus to the Imaging Plane—If the temporal focus optics are correctly aligned, it should be possible to obtain near-diffraction-limited two-photon illumination, or a thin sheet of illumination ~ 1 μm in thickness. We measure the performance of the temporal focusing by scanning a thin quantum dot layer (see Note 10) axially through the fixed illumination path. An example of optimal performance is shown in Fig. 10. An apparent thickness >1.5 μm (using the $60\times$, 1.2 NA water-immersion objective) indicates either a problem with optical alignment or a thicker than ideal test sample.

Achieving optimal temporal focus sectioning is contingent on proper alignment, yet how to achieve optimal alignment is not obvious. We find it useful to work backwards from the microscope, by following these steps:

1. Starting with the objective and dichroic in the Olympus microscope, we set a reference beam. This beam must be well collimated, since it will be used to set the axial position of several lenses in the system. We collimate our beam by adjusting a beam expansion telescope and measure its collimation by deflecting it off the optical table with a mirror. If the beam is made to travel several meters before hitting a wall, we can tell accurately if it's expanding or contracting. The beam diameter should be reasonably large (at least a centimeter), ideally with an adjustable iris to allow easy changing of the beam diameter. The back reflection of the reference beam from the objective should be very well aligned with the reference beam, indicating normal incidence on the back focal plane of the objective. Note that any back reflection which makes its way back into the femtosecond laser source will cause instability and prevent mode-locking, so we align just poorly enough to prevent this. With the objective removed, the reference beam should propagate in the same direction the objective points. We find it helpful to use a plumb line to mark a spot on the ceiling directly above the objective (assuming the objective is mounted vertically) and ensure that the center of the beam falls on this spot with/without the objective lens.
2. With a reference beam set, the next lens (working backwards from the objective, this is the $f = 500$ mm lens in Fig. 9a) can now be aligned. The axial position of the lens is correct when the combination of the lens and the objective produces a nearly collimated beam, i.e., the diameter of the beam on the ceiling mark is minimized (position the objective's z position near its operating position while making this alignment). The transverse position of the lens is correct when it does not deflect the reference beam; the reference beam should hit the same spot on the ceiling that it does with this lens and the objective removed. The tilt of this lens is correct when the back reflection from this lens points back along the reference beam. The curved surface of the lens is normally placed towards a collimated beam. Note that with temporally focused beams, it can be confusing whether or not a beam is "collimated" or converging/diverging. In this

- case, note that the beam in the sample is “temporally focused,” which at any given instant is equivalent to a point focus. Therefore, we can regard the beam between the $f=500$ mm lens and the objective as collimated and place the curved side of the $f=500$ mm lens facing this direction. With this lens in place, we can remove the objective and check if the reference beam still hits the reference mark on the ceiling. If it doesn’t, this means the beam was not well centered on the objective. Reset the reference beam, and repeat the alignment of this first lens.
3. Two more lenses ($f=100$ mm and $f=300$ mm in Fig. 9a) are now added by the same procedure: back reflection, non-deflection, and preserving collimation (*see* Note 14).
 4. Next we align our diffraction grating. Since some alignment processes are easier without the grating, we use a magnetic mount (Thorlabs, KB3X3) that allows us to switch the grating for a flat mirror. Since different wavelengths of light diffract off the grating at different angles, we deliver light to the grating with a mirror on a linear translation stage (Fig. 9a, d) very close to the grating to allow adjustment when we tune our femtosecond laser to different wavelengths. In practice, however, we almost always use this laser at 800 nm. To position the grating axially, we find it is sufficient to measure the distance from the grating to the next lens ($f=300$ mm in Fig. 9a) with a ruler, placing it one focal length away. To position the grating transversely, we center it on the reference beam before redirecting the reference beam to the mirror on the linear translation stage. We then set a new reference beam, roughly parallel to the old one but displaced to the side, bouncing off the mirror on the linear translation stage towards the grating. We tune the input angle of the grating by moving the linear translation stage and rotating the mirror mounted on it. We choose the input angle so that the first-order diffraction of the grating hits the next lens ($f=300$ mm in Fig. 9a) directly on center. We choose the tilt angle of the grating such that the output beam is normal to the grating face (*see* Note 15).

¹⁴When there is an even number of lenses (including the objective), this process works very well. When there is an odd number of lenses, the output of the objective diverges rapidly and deflection is very hard to judge. In this case, unscrew the objective during alignment, and restore it when it’s time to add the next lens.

¹⁵Pay attention to the blaze of the grating; there are two orientations of the grating which will work, but one will be substantially more efficient at reflecting the first-order diffracted beam.

5. With the grating positioned, we next place a cylindrical lens one focal length away from the grating, centered on the reference beam (CFL in Fig. 9a). This lens produces a horizontal line focus on the grating. Since the beam does not impact the grating at normal incidence, the line is only truly in focus at one point on the grating. However, the axial demagnification of the lens system between the grating and the sample is quite large (250,000 $\times$), so the effects of this defocus are negligible in the sample. As with previous lens alignment, the vertical position can be set by non-deflection, and the tilt can be set by back reflection. The horizontal position of this lens is unimportant, as long as it doesn't clip the beam.
6. Next we place a cylindrical beam expander in the reference beam (6.7 $\times$ CBE in Fig. 9a). The axial position of this expander is not critical, but the relative axial positions of the two cylindrical lenses in the expander are quite important. As before, non-deflection, back reflection, and collimation tests are used to insert these lenses in the reference beam. We make sure to position this expander close enough to the diffraction grating that enough room is left for the galvanometric mirror.
7. Positioning the galvanometric mirror may be the most difficult step in the process. We measure one focal length from the cylindrical focusing lens (in the direction away from the microscope, towards the reference beam) and place a reference object at this point, centered on the reference beam. We typically use a small-diameter Allen key attached to the end of an optical post. The location of this reference object is used to determine where the galvanometric mirror should be positioned. Once the reference object is set, we place the galvanometric mirror with its periscope assembly in its final position and send a new reference beam through the optical system. Even with a good reference object, finely aligning the galvanometric mirror is challenging, since the entire assembly must be moved to rotate the galvanometric mirror about a vertical axis, but this motion disrupts the reference beam passing through the galvo. We iterate, adjusting the rotation and resetting the reference beam several times.
8. With two-photon illumination optics aligned, we check the depth at which the two-photon beam reaches maximum intensity. We use a thin layer of quantum dots for this measurement (see Note 10) and illuminate the quantum dot

sample with 800 nm light while viewing its fluorescence on the EM-CCD. The layer has bumps and features that inform us when the camera emission path is focused on the quantum dots, and the fluorescent brightness of this informs us when the illumination path is focused on the quantum dots. Hopefully, these two depths match closely. If not, we move the camera axially using the attached linear translation stage (Thorlabs, DT25) to align the camera's focus with the illumination's focus.

For 3D PALM, the best localization precision is often obtained for molecules which are moderately out of focus. To bias activation towards slightly out-of-focus molecules, we translate the camera axially a small degree. The optimal level of defocus can be estimated by imaging a sample of gold fiducials, with a cylindrical lens in the imaging path. We start with the camera initially axially coaligned with the illumination then switch to a gold fiducial sample. We focus on the gold fiducials such that they appear maximally round and then axially translate the camera until they appear slightly elliptical.

3.3.6 Data Acquisition—Taking data follows the same scheme as Subheading 3.2.6, but with a few differences. During acquisition, we photoactivate the sample using 2PA illumination by scanning the galvanometric mirror at 500 Hz. As this speed is much higher than our acquisition rate (usually 10–13 Hz), we drive the galvo with the aid of a function generator (Stanford Research Instruments, DS340), finding it unnecessary to synchronize scanning with camera exposure. During acquisition we modify the 2P illumination power by rotating the half-wave plate placed in the automated rotation mount, as appropriate for the desired degree of photoactivation.

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Fig. 1.

Concept behind PALM. When labeled with a fluorescent probe and viewed through a conventional microscope, high spatial frequency detail in an object (*leftmost column*) is obscured, producing a diffraction-limited image (*middle left column*). PALM may be used to obtain a much higher-resolution image, if two criteria are met. First, the sample must be labeled with a photoswitchable probe and only a sparse subset of probes induced to activate and fluoresce in any given frame (*middle right column*). This “isolation” procedure alone is insufficient, as combining the recorded fluorescence from all N frames recovers the diffraction-limited image. However, if the optically resolved images of the individual molecules are also localized (*rightmost column*) with high precision, the aggregate image resulting from all such localizations may be combined to obtain a super-resolution image

Fig. 2.

Basic (2D) PALM system. **(a)** Front (*top*) and side (*bottom*) views, showing major items referred to in text including microscope frame, vibration isolation table, overhead shelf, and excitation sled. **(b)** Expanded schematic (*top*) and photograph (*bottom*) of excitation/activation lasers, associated optics, and optomechanics. Symbol key: *DC* dichroic mirror, *BS* beam-steering assembly, *ND* neutral density filter, *BE* beam expander, *SH* shutter, *TS* translation stage, *FL* focusing lens, *CL* collimating lens. Lasers are labeled according to wavelength, as referred to in the text. Lenses are drawn as *ellipses* in the schematic, reflective mirrors as *filled-in rectangles*. Note that DC1 and the 532 nm laser are not used for exciting PA-FPs and are thus not referenced in the text. Panel **(b)** is modified from York 2011, supplementary Fig. 1 with permission from Nature Methods

Fig. 3. Performance of commercial focus-lock system in reducing axial drift. A 100 nm gold fiducial marker stuck on a glass coverslip was localized repeatedly over a 15-min period. *Blue curve* indicates native axial drift; *red curve* demonstrates significant reduction in axial drift when the Mad City Labs C-Focus module is used

Fig. 4. Optomechanics for coupling cylindrical lenses to emission path of microscope. (a) Photograph and (b) accompanying schematic. Adapter 1 has external SM1 threads and internal C-mount threads and connects the 1.2× expander to the filter wheel that holds the cylindrical lenses. Adapter 2 is made up of two parts that mate together, one that has external C-mount threads and internal SM1 threads (for connecting the camera) and a second with external SM1 threads (for connecting the filter wheel). We find it useful to position a small support beneath the 1.2× expander to provide mechanical stability. A translation stage is used to position the imaging plane in an optimal position with respect to the excitation (see discussion in Subheadings 3.2.3 and 3.3.5)

Fig. 5.

Effect of cylindrical lenses with different focal length on imaging field and PSF. The same field ($z = 1 \mu\text{m}$ above the coverslip surface) was imaged conventionally and with 300, 150, and 100 mm focal length cylindrical lenses. Selected slices ($-2, -1, 0, 1,$ and $2 \mu\text{m}$ from the coverslip surface) from PSFs measured on the same gold bead are also displayed next to each imaging condition. Shorter focal lengths introduce greater distortion of the imaging field (particularly in horizontal direction) but introduce more astigmatic character in the PSF. Scale bar in *top left image*: $5 \mu\text{m}$

Fig. 6.

Images of 100 nm gold fiducial markers at indicated z position, taken on different coverslips with different tilt and thickness. *Arrows* indicate features that cannot be easily accounted for with a theoretical PSF, such as “wings” in horizontal or vertical directions or an asymmetric intensity distribution in the center of the PSF. Reproduced from York 2011, supplementary material with permission from Nature Methods

Fig. 7.

Example cross-correlation plots. Comparing the maximum cross-correlation between each slice of the calibration stack (Subheading 3.2.6) is useful in judging the suitability of the imaging conditions for 3D PALM. With no cylindrical lens (*top*), most axial slices of the fiducial marker resemble each other, resulting in large areas of high cross-correlation. This results in a PSF that will confuse the cross-correlation localization algorithm and cause poor axial localization. With an $f = 150$ mm cylindrical lens (*bottom*), the usable axial localization range is extended as there are fewer regions of high cross-correlation

Fig. 8.

Example of 3D PALM. Selected slices from a volumetric 3D PALM dataset of pa-mCherry1 lamin fusion proteins at indicated position above the coverslip surface highlighting two nuclei in fixed Cos7 cells. The sample was photoactivated with 2P line-scanning temporal focus as described in Subheading 3.3, resulting in a high volumetric localization density. XYZ pixel sizes are $60 \times 60 \times 60$ nm; Gaussian smoothing of 1 pixel was applied to the data. Sections of the nuclear membrane are resolved to better than 100 nm

Fig. 9.

Optical setup for 2PA of PA-FPs. **(a)** Optical schematic, including 1P optical sled (Fig. 1b), 2P optics, and coupling optics. Symbol key is as in Fig. 1b, with the following additions: *HWP* half-wave plate, *POL* polarizer, *PER* periscope assembly, *GAL* galvanometric scan mirror, *CFL* cylindrical focusing lens, *CBE* cylindrical beam expander, *GR* grating, *RM* removable mirror. *Dashed boxes* indicate setup components further detailed in photographs **(b)**–**(e)**. **(b)** 2P power control. **(c)** Periscope assembly that directs beam onto scanning mirror. **(d)** Cylindrical optics and diffraction grating. **(e)** Periscope and coupling optics that direct 2P and 1P illumination into laser port B. In **(b)**–**(e)**, *arrows* indicate direction of illumination through the setup. Panel **(a)** is modified from York 2011, supplementary Fig. 1 with permission from Nature Methods

Fig. 10.

Temporal focus optical sectioning. An 800 nm thick quantum dot layer was scanned in 100 nm axial steps through a line-scanning temporal focus illumination and the fluorescence recorded at each step. The normalized, integrated fluorescence at each frame is shown. Note the greatly reduced axial FWHM of 2P, line-scanning temporal focus illumination ($\sim 1.3 \mu\text{m}$) relative to wide-field, 488 nm illumination