Online Methods

Optical setup. A schematic of the dual-objective STORM setup is shown in Fig. 1a. Two infinity-corrected microscope objectives (Olympus Super Apochromat UPLSAPO 100x, oil immersion, NA 1.40) were placed opposing each other and aligned so they focus on the same spot of the sample. A piezoelectric actuator (Thorlabs DRV120) was used to control the axial position of the sample with nanometer precision. The 647 nm line from a Kr/Ar mixed gas laser (Innova 70C Spectrum, Coherent) and the 405 nm beam from a solid state laser (CUBE 405-50C, Coherent) were introduced into the sample through the back focal plane of the first objective using a customized dichroic mirror that worked at an incident angle of 22.5° (Chroma). A translation stage allowed the laser beams to be shifted towards the edge of the objective so that the emerging light reached the sample at incidence angles slightly smaller than the critical angle of the glass-water interface, thus illuminating only the fluorophores within a few micrometers of the coverslip surface. The fluorescence emission was collected by both objectives. After passing through long-pass filters (HQ665LP, Chroma), the two parallel light rays from the two objectives were each focused by a 20 cm achromatic lens, cropped by a slit at the focal plane, and then separately projected onto two different areas of the same EMCCD camera (Andor iXon DU-897) using two pairs of relay lenses. Astigmatism was introduced into the imaging paths of both objectives using a cylindrical lens so that the images obtained by each objective were elongated in x and y for molecules on the proximal and distal sides of the focal plane (relative to the objective), respectively. A band-pass filter (ET700/75m, Chroma) was installed on the camera. See **Supplementary Protocol 1** for more details.

Sample preparation. BSC-1 and COS-7 cells were plated on 18-mm diameter, #1.5 uncoated glass coverslips at a confluency of ~20%. After 16-24 hours, the cells were fixed and labeled following previously developed protocols for ultrastructural studies of actin cytoskeleton[20-22] (See **Supplementary Protocol 2** for details). Briefly, the cells were initially fixed and extracted for 1-2 min using a solution of 0.3% glutaraldehyde and 0.25% Triton X-100 in cytoskeleton buffer (CB: 10 mM MES pH 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, and 5 mM $MgCl_2$), and then post-fixed for 10 min in 2% glutaraldehyde in CB. The sample was treated with freshly-prepared 0.1% sodium borohydride for 7 min to reduce background fluorescence. For vinculin staining (when needed), the sample was first blocked with 3% BSA and 0.5% Triton X-100, and then stained with rabbit monoclonal vinculin antibodies (Invitrogen 700062) followed by Cy3-labled goat anti-rabbit secondary antibodies (Invitrogen A10520). Actin filaments were labeled with Alexa Fluor 647-phalloidin (Invitrogen A22287) overnight at 4 °C. A concentration of ~0.5 μM phalloidin in phosphate buffered saline (PBS) was used. To minimize the dissociation of phalloidin

from actin, the sample was briefly washed once with PBS and then immediately mounted for STORM imaging.

For drug-effect studies, cells were incubated with culture media containing either 0.5 μM cytochalasin D (Sigma-Aldrich), 0.25 μM latrunculin A (Invitrogen), or 50 μM (−)-blebbistatin (the active enantiomer; Sigma-Aldrich) at 37°C for 1 hour, and then fixed and labeled as described above.

The STORM imaging buffer for fixed cells was PBS with the addition of 100 mM cysteamine, 5% glucose, 0.8 mg/mL glucose oxidase (Sigma-Aldrich), and 40 μg/mL catalase (Roche Applied Science). ~4 μL of imaging buffer was dropped at the center of a freshly-cleaned, #1.5 rectangular coverslip (22 mm by 60 mm), and the sample on the 18-mm diameter coverslip was mounted on the rectangular coverslip and sealed with nail polish.

Image data acquisition. The sealed sample was mounted between the two opposing objectives. The 647 nm laser was used to excite fluorescence from Alexa Fluor 647 molecules. Prior to acquiring STORM images, we first used relatively weak 647 nm light (~0.05 W/cm^2) to illuminate the sample and recorded the conventional fluorescence image before any substantial fraction of the dye molecules were switched off. We then increased the 647 nm light intensity (to ~2 kW/cm^2) to rapidly switch the dyes off for STORM imaging. The 405 nm laser was used to reactivate the fluorophores from the dark state back to the emitting state[23]. The power of the 405 nm laser (0-1 W/cm^2) was adjusted during image acquisition so that at any given instant, only a small, optically resolvable fraction of the fluorophores in the sample were in the emitting state. The EMCCD camera acquired images from both objectives simultaneously at a frame rate of 60 Hz. Typically, ~90,000 frames were recorded to generate the final super-resolution image of the actin ultrastructure. Recording of more frames (e.g., 230,000 frames for Fig. 2) further improved the image quality at the expense of longer imaging time.

Image data analysis. The recorded STORM data were first split into two movies, each of which comprises a sequence of images obtained by one of the two objectives. Each movie was first analyzed separately according to previously described methods[12]. The centroid positions and ellipticities of the single-molecule images provided lateral and axial positions of each activated fluorescent molecule, respectively. The molecular positions obtained by the second objective were mapped to the coordinates of the first objective through a transformation based on corresponding features (control points) in both images[24]. The mapped data from the two objectives were then compared frame-by-frame: molecules that were switched on within one-frame of time and that were within ~50 nm to each other in the mapped *x*-*y* plane were identified as the same emitting

molecule detected by both objectives. Non-matching molecules were discarded. For each pair of matched molecules observed by the two objectives, the availability of two z-positions obtained through the two objectives provides a novel means to identify abnormalities and cancel noise. Since the focal planes of the two opposing objectives coincided, a molecule on the side of the focal plane proximal to one objective would be on the distal side for the other objective. Therefore, its image would appear elongated in x through one objective but elongated in y through the other objective. A real change in the z position would cause anti-correlated changes in ellipticity measured through the two objectives (Fig. 1d, green and blue arrows). On the other hand, abnormalities and noise would tend to cause correlated changes in ellipticity. For example, when two close-by molecules with overlapping images were misidentified as a single molecule, the resultant images through both objectives would appear elongated in the same direction along the line connecting the two molecules (Fig. 1d, magenta arrow). Likewise, any x-y drift of the stage or the camera would also cause elongation in the same direction. These correlated changes in ellipticity resulted in apparently different z positions obtained through the two objectives (Δz). The value of Δz can thus be used to identify and reject abnormalities. These abnormalities (identified by $\Delta z > \sim 100$ nm, which is substantially larger than the axial resolution of a single objective) amounted to ~10% of all identified molecules. For molecule pairs that match well with each other in all four (spatial + temporal) coordinates, the final coordinates were determined as the average of the mapped coordinates from the two objectives, weighted by the width of the image and number of photons obtained by each objective. This averaging procedure further reduced noise caused by errors, such as the correlated changes in ellipticity described above. The final super-resolution images were reconstructed from these molecular coordinates by depicting each location as a 2D Gaussian peak. Analysis software is available as **Supplementary Software**.

To characterize the localization precision, we used fixed cell samples sparsely labeled with Alexa Fluor 647. Relatively strong activation conditions were used such that each Alexa Fluor 647 molecule was activated multiple times during the image acquisition time and gave a cluster of localizations due to the repetitive activation. The sparse labeling condition allowed us to readily identify these clusters of localizations. Localizations from many such clusters within ~150 nm of the focal plane were aligned by their center of mass to generate the localization distribution reported in Fig. 1b. The localization precision determined from these distributions was ~4 nm (SD) in the x-y directions and ~8 nm (SD) in the z direction, respectively. The variation in localization precision across this region was small (within 15% of the average value). The localization precision determined from the sparsely labeled sample is a good representation for the densely labeled actin

samples as the parameters relevant for the localization precision, such as the number of photons detected from individual molecules and the background fluorescence signal, were measured to be the same for both sparsely and densely labeled samples.

For STORM imaging of the densely labeled actin samples, relatively weak 405 nm activation intensities were used during image acquisition. This led to a typical linear localization density of 1 localization per 4 nm along individual actin filaments for ~90,000 frames, which corresponds to a Nyquist Criterion based resolution of 8 nm, smaller than the 9 nm lateral and 19 nm axial resolutions determined above. As we had not exhausted all of the label molecules by the end of image acquisition, the number of localizations can be further increased by increasing the imaging time.

For the characterization of the widths of individual actin filaments, we chose short (~200 nm), straight segments of filaments in cells along which no crossing or branching of filaments were observed. Analysis of eight such segments yielded FWHM widths of 12 ± 2 nm.

References

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