Expansion Enhanced Nanoscopy

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Supplementary information, Data S1, Methods and Materials

Reagents and antibodies

For cell culture, Dulbecco's Modified Eagle Medium (C11995500BT), fetal bovine serum (10099141), antibiotics (penicillin and streptomycin; 1514-122), nonessential amino acid (11140050) and Dulbecco's Phosphate-Buffered Saline (14190250) was purchased from Life Technologies. For immunostaining of cultured cells, primary antibodies are listed as follows: mouse anti-alpha tubulin (ab74696, Abcam), mouse anti-acetylated tubulin（T6793, Sigma）, rabbit anti-detyrosinated tubulin (ab48389, Abcam), rat anti-tyrosinated tubulin (MA1-80017), rabbit anti-clathrin (ab21679, Abcam), rabbit anti-Nup133 (ab155990, Abcam), rabbit anti-Homer1 (160003, Synaptic Systems) and mouse anti-Bassoon (ab82958, Abcam). Organic dyes and Qdots conjugated secondary antibodies were purchased from Life Technologies, listed as follows: Alexa Fluor 488 conjugated donkey anti-rabbit antibody (A-21206), Alexa Fluor 488 conjugated goat anti-mouse antibody (A-11001), Alexa Fluor 594 conjugated goat anti-mouse antibody (A-11032), Alexa Fluor 594 conjugated goat anti-rabbit antibody (A-11072), Qdot Streptavidin Sampler Kit (Q10151MP), Qdot605 conjugated donkey anti-rabbit antibody (Q-11001MP), Qdot605 conjugated donkey anti-mouse antibody (Q-11401MP), Qdot655 conjugated goat anti-rat antibody (Q-11621MP), Qdot705 conjugated goat anti-mouse antibody (Q11062MP) and

Qdot705 conjugated goat anti-rabbit antibody (Q11461MP). Streptavidin functionalized dyes and Qdots were obtained from Life Technologies. 8% paraformaldehyde (157-8) was obtained from Electron Microscopy Sciences (EMS). Triton X-100 (T8787), sodium borohydride (71320), poly-L-lysine (P4707) and biotin-amidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester (biotin-NHS) (B3295) were obtained from Sigma-Aldrich, bovine serum albumin (BSA) was purchased from Jackson ImmunoResearch Laboratories, illustra NAP-5 colums seephadex G-25 DNA grade (17-0853-02) were purchased from GE Healthcare. Expansion microscopy related reagents are listed below: 50% glutaraldehyde (16200, EMS) was obtained from Electron Microscopy Sciences, Methacrylic acid N-hydroxy succinimidyl ester (MA-NHS) (730300), 4-hydroxy 2,2,6,6-tetramethylpiperidin-1-oxyl (4-hydroxy-TEMPO) (176141), acrylamide (A9099), N, N'-methylenebisacrylamide (M7299), sodium acrylate (408220), Tetramethylethylenediamine (T22500), ammonium persulfate (a3678), guanidine hydrochloride (G3272), proteinase K (P4850) and poly-L-lysine solutions were obtained from Sigma-Aldrich. 50 mm glass bottomed dish (14026) was obtained from Ted Pella, Inc..

Cell culture

The HeLa (Provided by professor Wei Guo, University of Pennsylvania), U2OS cells (Cell Bank of Chinese Academy of Sciences, China) were cultured in Dulbecco's Modified Eagle Medium (Life Tech.) supplemented with 10% fetal bovine serum (Gibco) containing penicillin and streptomycin (15140-122, Life Technologies). BS-C-1 cells (BeNa Culture Collection, China) were cultured in Modified Eagle Medium basic (Life Tech.) supplemented with 10% fetal bovine serum (Gibco) containing penicillin and streptomycin (15140-122, Life Tech.) and nonessential amino acid (NEAA,

11140-050, Life Tech.). Cells were plated on 35 mm glass bottomed dish (Shengyou, China) with 10 mm micro-well for 2 days at 37℃ environment with 5% CO_2.

Immunostaining of cultured cells

For general labelling, cells were harvest at the confluence around 80% and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. For optimal microtubule labelling, cells were extracted with extracting buffer containing 0.1 M PIPES, 1 mM EGTA, 1 mM $MgCl_2$, 0.2% Triton X-100 for 1 min before fixation. After brief washing in PBS, cells were reduced with 10 mM sodium borohydride for 7 min. After reduction, cells were permeabilized with 0.5% Triton X-100 for 5 min at room temperature. Cells were then washed again and blocked with blocking buffer containing 5% (w/v) bovine serum albumin (BSA) and 0.5% Triton X-100 for 30 min. Specimens were then incubated with primary antibodies in blocking buffer for 1 hour at room temperature, washed three times with PBS, and incubated for 1 hour with organic dyes or Qdots conjugated secondary antibodies. Cells were extremely washed with PBS and stored at 4℃. In particular, in order to increase the fluorescence intensity after expansion, we incubated the sample with biotin-conjugated primary antibodies, and then incubated with corresponding organic dye conjugated secondary antibodies. After expansion, gels were incubated with dye-conjugated streptavidin at room temperature for 8-12 h, followed by washing at 37℃ for 2-3 h in PBS. After immunostaining, organic dye labeled specimens were treated with 0.25% GA for 10 min at room temperature. For Qdots labeled cellular structures, specimens were treated with 0.25% GA for 30 min at room temperature, followed by washing three times with PBS.

Immunostaining of mouse brain tissue

For typical staining, mouse brain tissues were washed three times with PBS and sequentially penetrated with 0.5%, 0.8% Triton X-100 for 10 min each. Specimens were then incubated with the blocking buffer containing 3% BSA and 0.1% Triton X-100 for 1h at room temperature. After incubation, tissue slices were then incubated with primary antibodies in blocking buffer for 12 ~16 h at room temperature, washed three times with blocking buffer for 1 h, and incubated with secondary antibodies for 12~16 h at room temperature. Specimens were extremely washed with PBS, and treated with 1mM MA-NHS in PBS for 1 h at room temperature, followed by three washes with PBS.

Preparation of biotin-conjugated antibodies

As reported before,[1] 12 μL of 1 M sodium bicarbonate solution were added to 100 μL of 1mg/mL antibodies to change the pH to about 8.0. After mixing the solution, 10 μL of 1 mM biotin-NHS were added to the mixture, followed with incubation at room temperature for 30 min. Biotinylated antibodies were separated from free biotin-NHS with illustra NAP-5 columns and stored at 4 °C before use.

Gelation, digestion, and expansion of cell specimens

Expansion microscopy was conducted as described before.[2] Generally speaking, monomer solution (1 x PBS, 2 M NaCl, 2.5% acrylamide, 0.15% N,N'-methylenebisacrylamide, 8.625% sodium acrylate) was mixed, frozen in aliquots, and thawed before use. Concentrated stocks of 10% ammonium persulfate (APS) and 10% tetramethylethylenediamine (TEMED) were diluted in monomer solution to final concentration of 0.2% in gelation solution. Cells were incubated with monomer solution for 1 min at room temperature before gelation. About 100 μL gelation solution were added in the central well.

Gelation was allowed to proceed at 37℃ for 30 min and placed in digestion buffer (1 x TAE buffer, 0.5% Triton X-100, 0.8 M guanidine HCl, 8 units mL^{-1} Proteinase K) for 30 min at 37℃. The gels were removed from the digestion buffer to deionized water to expand. Water was changed every 30 min, until the size of the sample plateaued.

Gelation, digestion, and expansion of mouse brain tissue

Fixed mouse brain slices were incubated with monomer solution at 4℃ for 45 min at room temperature before gelation, and gelled with gelation solution containing 4-hydroxy-TEMPO at a concentration of 0.01% (w/w) to inhibit gelation during diffusion of the monomer solution into tissue sections. The specimens were allowed to gel for 2h at 37 ℃, and digested in the digestion buffer for 2.5 h at 37℃. The samples were removed from the digestion buffer and placed in deionized water to expand.

Attachment of the gels to poly-L-lysine coated surface

40 mm glass bottomed dishes were treated with plasma for 2 min, and coated with 0.1mg/ml poly-L-lysine for 10 min at room temperature. Dishes were dried with nitrogen flow. Expanded samples could be stably attached to the glass surface and maintain immobility for a long time data collection.

Imaging and data processing

STED imaging was performed using a commercial STED microscope (TCS SP8 STED 3X, Leica Microsystems, Germany) equipped with a HCX PL APO x 100/1.40 NA objective. AF 488 labeled samples were excited with white laser at the wavelength of 488 nm, and AF594 labeled samples were

excited with white laser at the wavelength of 585 nm. STED laser for AF 488 was continuous wave at the wavelength of 594 nm, and the wavelength of STED laser for AF 594 was 775 nm pulsed laser. All images were acquired with the LAS AF software (Leica).

Wide field images for SOFI reconstruction were recorded using a commercial inverted Nikon Ti-E TIRF microscope, equipped with APO x 100/1.49 NA objective and EMCCD (Andor, DU-897). A 1.5 x telescope was used to reduce pixel size to ~106 nm. A 488 nm laser (Coherent) was used to excite the Qdots labelled samples. Blinking statistics were performed using home-written Matlabs 2013a code (Mathworks Inc., USA). For subcellular SOFI reconstruction, the image sequence drift was corrected by a subpixel drift correction algorithm,[1, 3] then the 2nd-order cross-cumulant SOFI analysis was implemented with shortest lag time using localizer software package in Igor Pro (WaveMetrics). Next, Richardson-Lucy deconvolution algorithm (Igor Pro) with five iterations was used for further resolution improvement. For fair comparison, the pixel sizes of averaged wide field images were interpolated the same as the different-order SOFI images using ImageJ (NIH, USA).

Registration of pre-expansion and post-expansion correlative images was carried out in the open-source software Elastix. In this method, both rigid and nonrigid transformations were used to determine the expansion factor and distortion in expansion microscopy.

Supplementary information, Figure S1

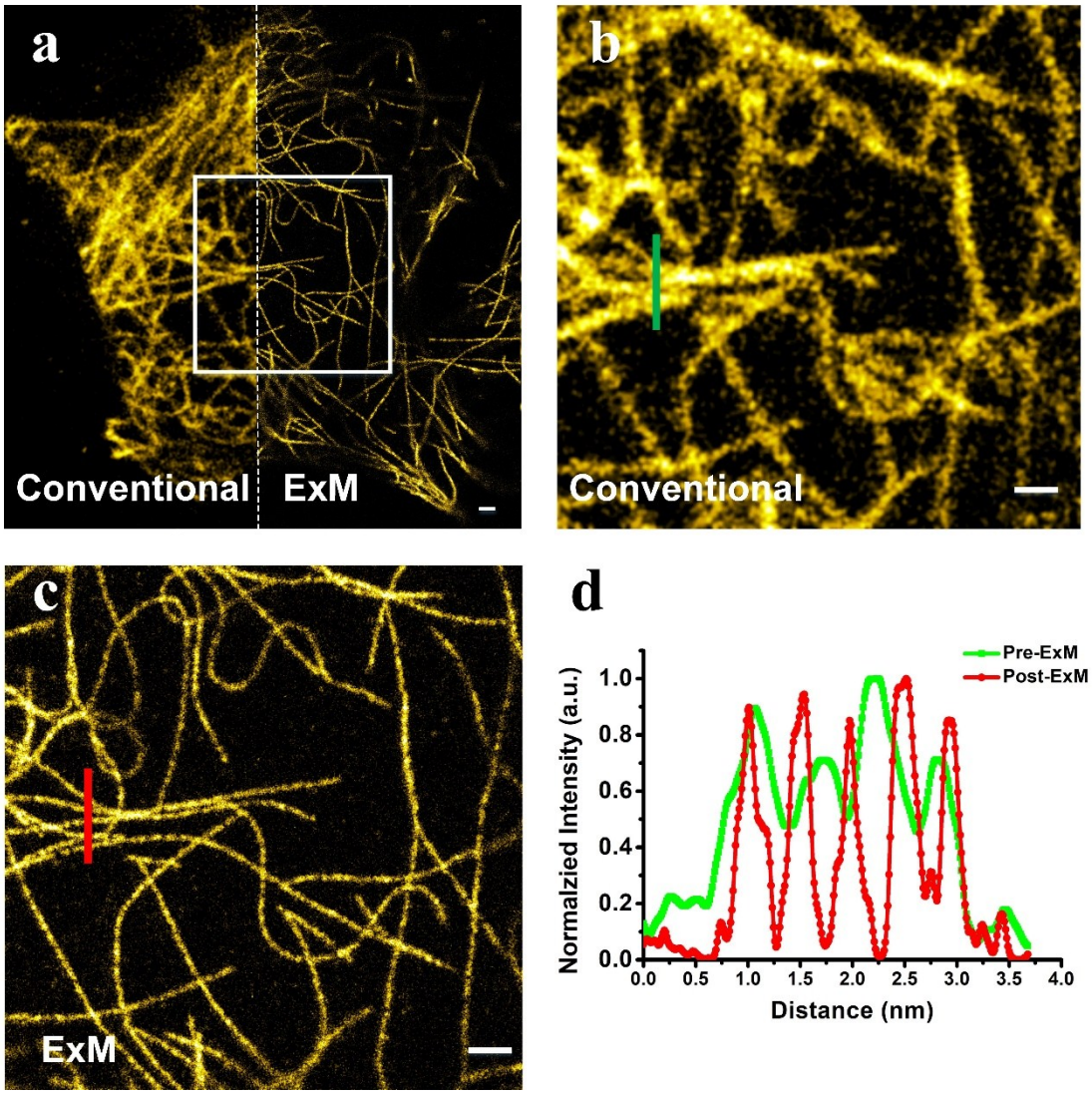

Figure S1 Images of Alexa Fluor 594 labelled microtubules in BS-C-1 cell line with different imaging techniques

(a) Cells were immunostained for alpha tubulin with conventional antibodies and expanded according to the standard expansion microscopy protocol. Confocal images of pre-expansion images (left) and partially overlaid with corresponding post-expansion images (right). (b) Zoomed-in pre-expansion confocal views of boxed region in (a). (c) Zoomed-in post-expansion confocal views of boxed region in (a). (d) Line profiles of the green line in b and corresponding red line in (c). Scale bar: (a-c) 1 μm.

Supplementary information, Figure S2

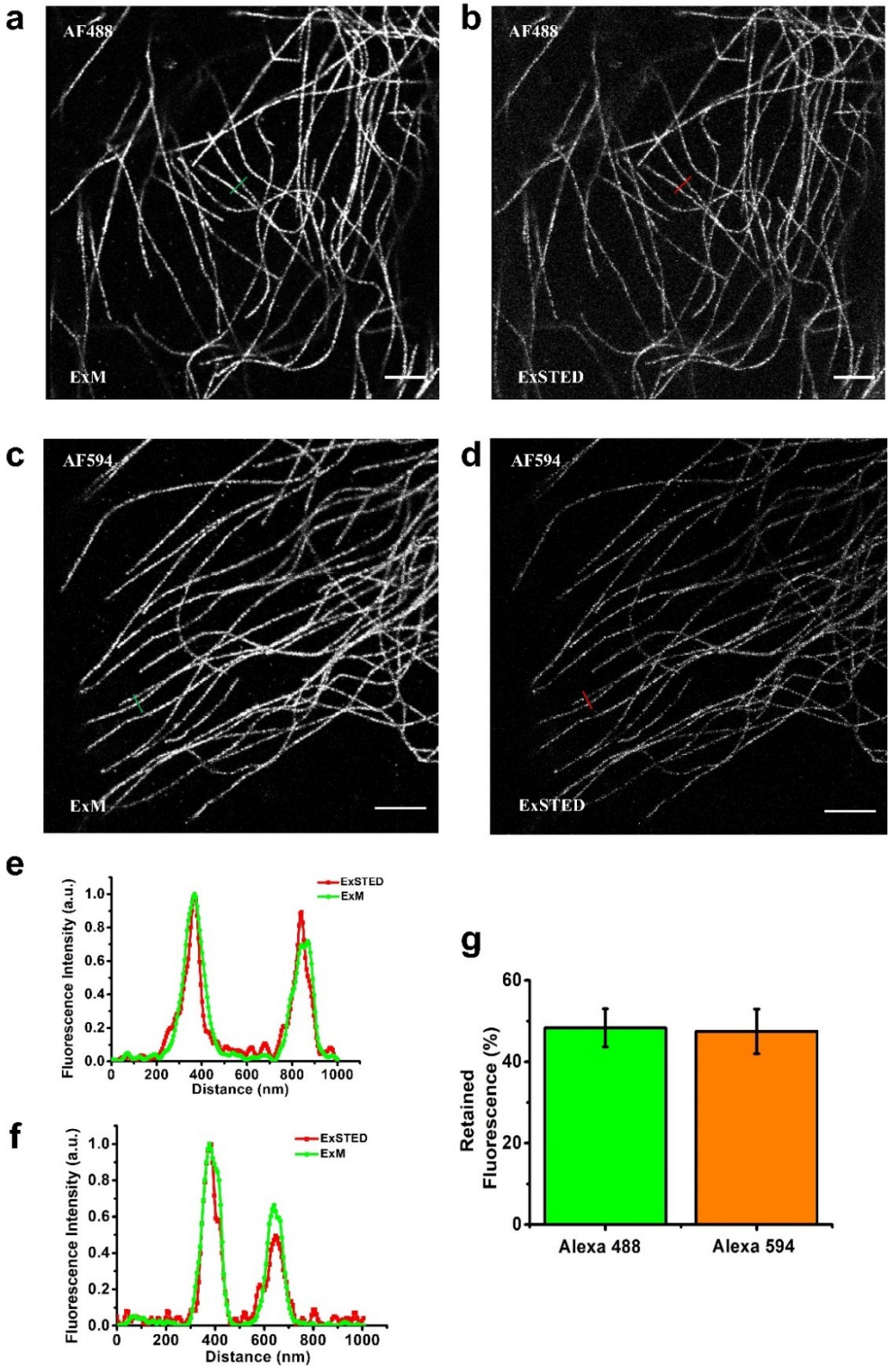

Figure S2 Images of organic dye labelled microtubules in BS-C-1 cell line after expansion microscopy

(a) Cells were immunostained for alpha tubulin with AF488 conjugated antibodies and expanded

according to the standard expansion microscopy protocol. Confocal images of expanded specimen. (b) ExSTED images of the same view in (a). (c) Cells were immunostained for alpha tubulin with AF594 conjugated antibodies and expanded according to the standard expansion microscopy protocol. Confocal images of expanded specimen. (d) ExSTED images of the same view in (c). (e) Line profiles of the green line in (a) and corresponding red line in (b). (f) Line profiles of the green line in (c) and corresponding red line in (d). (g) Retention of fluorescence for Alexa Fluor 488 and Alexa Fluor 594 labelled samples after ExM treatment. Scale bar: (a-d) 2 μm.

Supplementary information, Figure S3

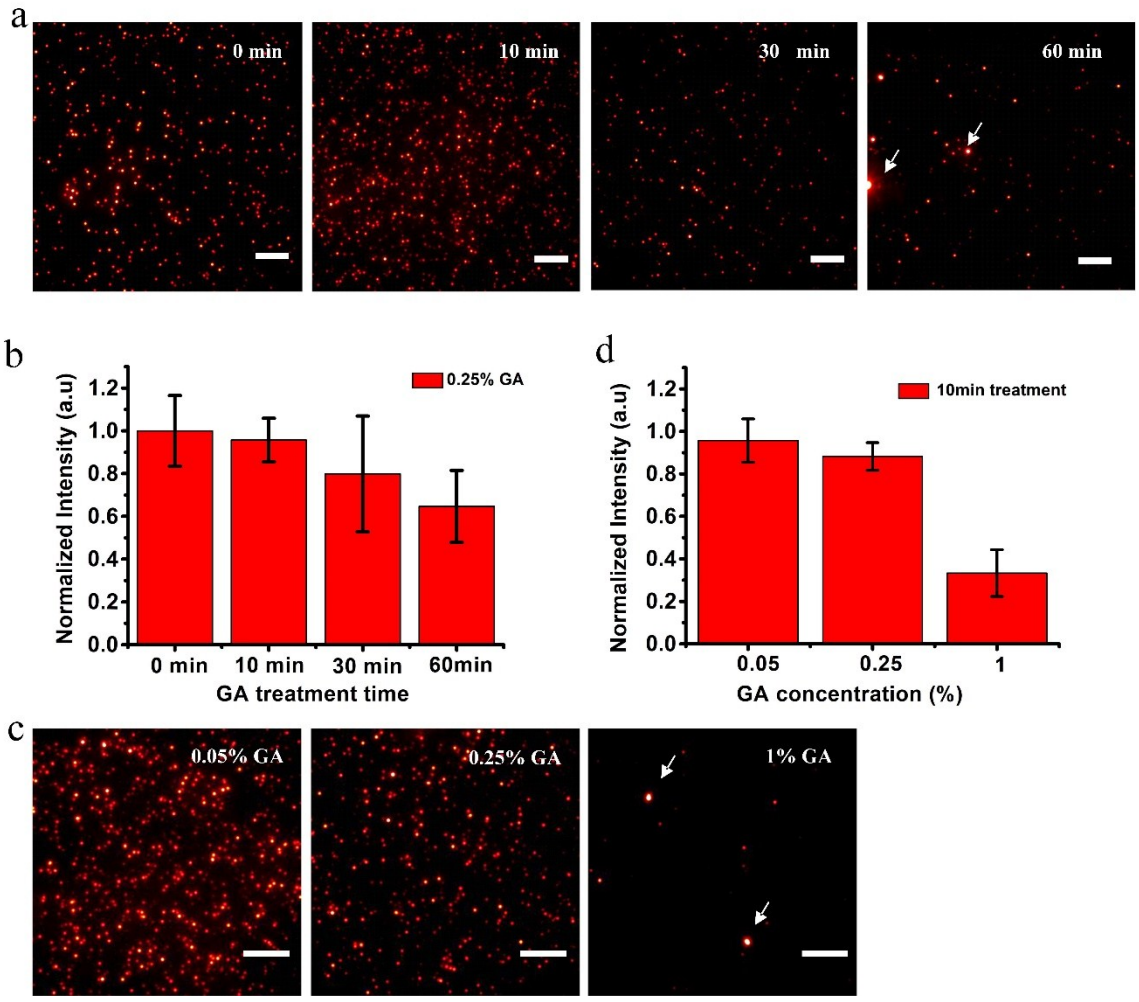

Figure S3 Effect of Glutaraldehyde (GA) on quantum dots (Qdots) *in vitro*

Qdots nanoparticles were incubated with GA at different conditions *in vitro*. (a) Qdot705 nanoparticles were treated with GA at 0.25% concentration for 0 minutes, 10 minutes, 30 minutes, and 60 minutes. Image acquisition was performed under the same conditions. After 60 minutes, obvious fluorescence intensity reduction and aggregation appeared. The white arrows indicate the two of the large aggregations in the field of view. (b) Quantitative analysis of fluorescence signal change in (a). (c) Qdot705 nanoparticles were treated with GA for 10 min at different concentration, including 0.05%, 0.25%, 1%. Image acquisition was performed under the same conditions. When treated with 1% GA, obvious fluorescence intensity reduction and aggregation appeared. The arrows indicate two aggregations in the field view. (d) Quantitative analysis of fluorescence signal change in (c). Scale bar: (a, c) 5 μm.

Supplementary information, Figure S4

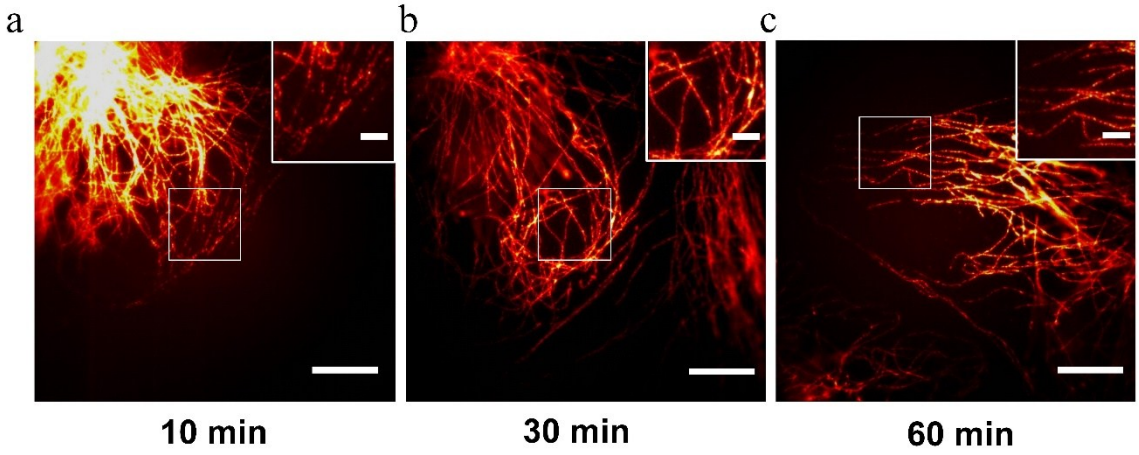

Figure S4 Wide field images of post-expansion microtubules labeled with Qdot705

BS-C-1 cell were immunostained for alpha tubulin with Qdot705, treated with 0.25% GA for different time, and processed for expansion microscopy as described before. Information of expanded specimens was obtained with wide field imaging. (a) With GA treatment for 10 minutes, the structure of microtubules was discontinuous. (b) With GA treatment for 30 minutes, the structure of microtubules is continuous and complete. (c) With GA treatment for 60 minutes, the microtubule structure is continuous. Scale bar: (a-c) 5 μm, scale bars in the corresponding inserted images were 1 μm.

Supplementary information, Figure S5

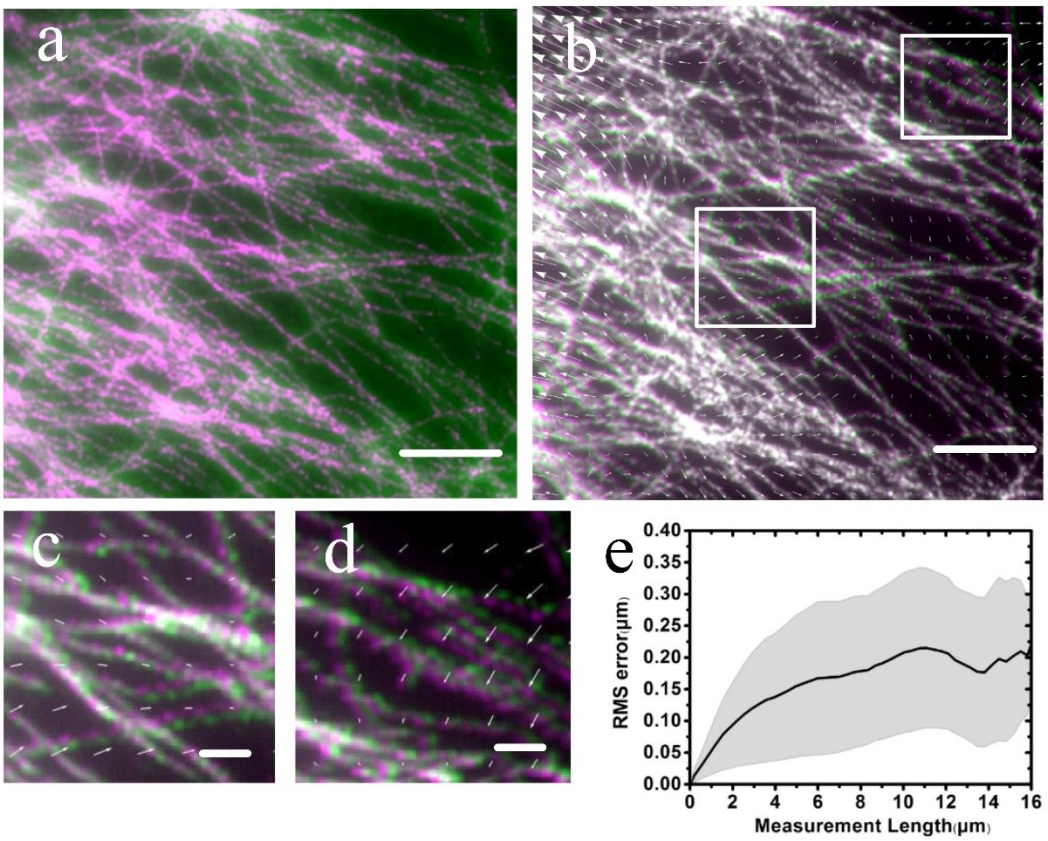

Figure S5 Comparison of pre-expansion and post-expansion images recorded by wide field imaging for a region of Qdot705 labeled microtubules in BS-C-1 cell line.

(a) The superposition of the image before expansion (magenta) and after expansion (green) with calibration using similar registration. (b) Use of a non-rigid overlay of the expanded image (magenta) after B-spline registration and the expanded image (green) after rigid registration. The arrows indicate the direction of rotation and the relative change in magnification required to completely match the two images. (c, d) Magnified images of the white box area in (b). (e) Quantitative analysis of changes in root mean square error versus measured distance in (b). All distances and scales are the same as those before expansion. Scale size: (a, b) 2 μm, (c, d) 0.5 μm.

Reference

1 Chen X., Li R., Liu Z. et al. Small Photoblinking Semiconductor Polymer Dots for Fluorescence Nanoscopy. *Adv. Mater.* 2017; **29**:1604850.

2 Chozinski T.J., Halpern A.R., Okawa H. et al. Expansion microscopy with conventional antibodies and fluorescent proteins. *Nat. Methods* 2016; **13**:485-488.

3 Guizar S.M., Thurman S.T., Fienup J.R. Efficient subpixel image registration algorithms. *Opt. Lett.* 2008; **33**:156-158.