Electronic Supplementary Information

Improving resolving ability of expansion microscopy by varying crosslinker concentration

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Material and methods:

Reagents.

All the antibodies were purchased from Abcam (Cambridge, UK) including anti-tubulin antibody (ab6160), anti-clathrin antibody (ab21679), anti-TOMM20 antibody (ab186734), goat anti-rat IgG (ab150165) and goat anti-rabbit IgG (ab150077). Paraformaldehyde (8%) and glutaraldehyde (8%) were bought from Hede Biotechnology (a distributor of Electron Microscopy Sciences, Beijing, China). Piperazine-N,N′-bis(2-ethanesulfonic acid) sesquisodium salt (PIPES), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and magnesium chloride ($MgCl_2$) were supplied by Sangon Biotech (Shanghai, China). Blocker BSA, anhydrous dimethyl sulfoxide (DMSO) and low-melting point agarose were obtained from Thermo Fisher Scientific (Waltham, MA USA). Sodium borohydride ($NaBH_4$), Triton X-100 and methacrylic acid N-hydroxysuccinimide ester (MA-NHS) were purchased from Sigma-Aldrich (St. Louis, MO USA). MA-NHS was resuspended in anhydrous DMSO at a concentration of 50 mg/mL, aliquoted and stored at -20 ℃. Tris-HCl buffer (pH 8), phosphate-buffered saline (PBS), sodium chloride and acrylamide were supplied by Sangon Biotech. Sodium acrylate, N,N′-methylenebisacrylamide (MBAA), ammonium persulfate (APS) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich. Proteinase K was obtained from New England Biolabs (Ipswich, MA USA). Deionized water was prepared by Milli-Q Ultrapure Water System (Merck KGaA Darmstadt, Germany).

Immunostaining of cultured cells.

HeLa cells were cultured on glass bottom dishes in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and were maintained at 37 ℃ environment with 5% CO_2. For tubulin, Cells were briefly washed with PBS and extracted with PEM buffer (100 mM PIPES, 1 mM EDTA and 1 mM $MgCl_2$ (pH 7)) containing 0.5% Triton X-100 for 30 s immediately before fixation.

Cells were fixed with a mixture of 3.2% formaldehyde and 0.1% glutaraldehyde (PFA/GA) in PEM buffer for 10 min, followed by reduction with 0.1% $NaBH_4$ for 5 min. For clathrin, cells were fixed with 3.2% formaldehyde in PBS for 10 min. Then specimens were washed in PBS three times for 5 min each and incubated with blocking/permeabilization buffer (3% BSA, 0.5% Triton X-100, 1× PBS) for 30 min. Specimens were stained with primary antibodies at a concentration of ~10 μg/mL in blocking/permeabilization buffer for 2 h and then were washed three times in PBS. Then specimens were incubated with fluorophore-conjugated second antibodies at a concentration of ~20 μg/mL in blocking/permeabilization buffer for 1 h and then were washed three times in PBS.

Anchoring treatment, gelation, digestion and expansion.

Gel solutions were modified from Edward S. Boyden's recipe. Monomer solutions (1× PBS, 2 M NaCl, 8.625% (w/w) sodium acrylate, 2.5% (w/w) acrylamide, different concentrations of N,N′-methylenebisacrylamide) were prepared by mixing stock solutions (10× PBS, 5 M NaCl, 38% (w/w) sodium acrylate, 50% (w/w) acrylamide, 2% N,N′-methylenebisacrylamide). Amount of crosslinker (N,N′-methylenebisacrylamide) was set from 0.04% to 0.2%. Monomer solutions were frozen in aliquots at -20 ℃ and thawed before use. For anchoring, stained cells were incubated with 1 mg/mL MA-NHS overnight at room temperature, and then were washed three times with PBS. Concentrated stocks (10% w/w) of tetramethyethlenediamine (TEMED) accelerator and ammonium persulfate (APS) initiator were sequentially added to monomer solution to concentration of 0.2% (w/w). The mixed solution was quickly added to the well of confocal dish and covered with plastic wrap to isolate from air. After 1 min at 4 ℃, the confocal dish was placed at 37 ℃ for 3 h for gelation.

Proteinase K was diluted 1:100 to 8 units/mL in digestion buffer (50 mM Tris-HCl buffer (pH 8), 1 M NaCl, 1 mM EDTA, 0.5% Triton X-100). In order to improve the permeation of proteinase K to the embedded cells, edge of the hydrogel was cut carefully.

The edge-cut hydrogel was incubated with digestion solution for 4 h at 37 ℃. Then the digestion solution was aspirated from the confocal dish and deionized water was added. Water was exchanged every 2 h until the hydrogel expanded to plateaus.

Image acquisition and date analysis.

All images were acquired on A1 MP multiphoton confocal microscope (Nikon Tokyo, Japan). Pre-expansion image was acquired with 60× 1.4 NA oil immersed objective and post-expansion image was acquired with 60× 1.2 NA objective in water. If needed, expanded sample was fixed on confocal dish with 2% low-melting point agarose. For finding the same cell in pre- and post-expansion sample, we used a pre-expansion image captured with 10× objective as a 'map'. We imaged the pro-expansion sample with 10× objective and find the cells. Then we imaged the cells with 60× objective.

Estimating fluorescence retention in digestion step: To get the same size, we immersed samples in digestion buffer for two times before each imaging. We captured z-series of the sample by confocal microscopy with maximal pinhole. After digestion, we imaged the same region with the same parameters. We calculated z-axis profile of the stack with ImageJ (Image → stacks → Plot Z-axis Profile) and selected the slice with maximal value for further measurement.[1] Cell regions were selected with image segmentation (Image → Adjust → Threshold → Set) and mean gray values of cell regions were measured (Analyze → Measure) in ImageJ. Fluorescence retention was calculated with the measurement before and after digestion images. If needed, subtract the background.

Measuring expansion factor and root-mean-square (RMS) error: Expansion factors were determined by measuring the distance between two landmarks in both pre- and post-expansion images with ImageJ (Analyze → Measure) and calculating the ratio of the measurements. Then, similarity transformation and B-spline transformation were performed in elastix with the method descripted by Jo. Vaughan's group.[2,3] Deformation vector field and RMS error were acquired with a Mathematica script written by them.[3]

Measuring microtubule full width at half maximum (FWHM): Intensity profiles were measured over a line perpendicular to microtubule orientation by ImageJ (Analyze → Plot Profile). Then, the intensity data were fit to a Gaussian function with ImageJ (Analyze → tools → Curve Fitting → Gaussian → Fit) and FWHM was calculated from the Gaussian fitting.

Measuring clathrin coated pits (CCPs) radii: we divided CCP into eight equal parts with four straight lines through the center of CCP. Intensity profiles of the straight lines were obtained with ImageJ. Then, the intensity data were fit to a double Gaussian function in Matlab. CCPs radii were calculated from the two peaks.

Richardson–Lucy algorithm: images were deconvolved in MatLab with the code as below:

```
>> A=imread('FWHM.tif');
>> B=im2double(A);
>> PSF = fspecial('gaussian',6,3);
>> C=deconvlucy(B, PSF);
>> D=im2uint16(C);
>> imtool(uint16(D))
```

Supplementary Figures

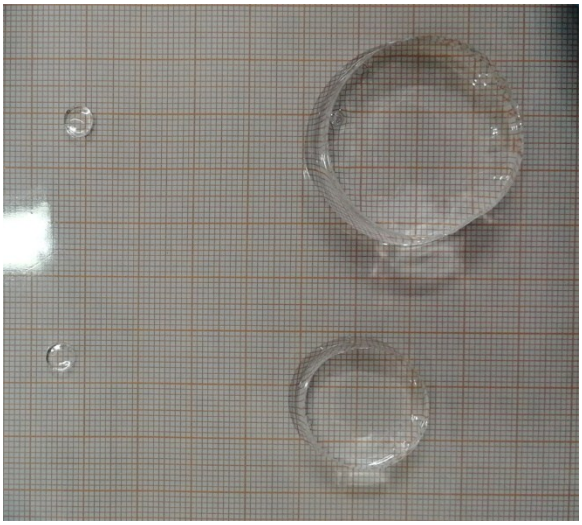

Fig. S1 Comparing expansion capacity of hydrogels with different crosslinker concentrations. The same size swellable hydrogels (left) expand to 6.5 times (upper right, with 0.06% MBAA) and 4.5 times (lower right, with 0.15% MBAA).

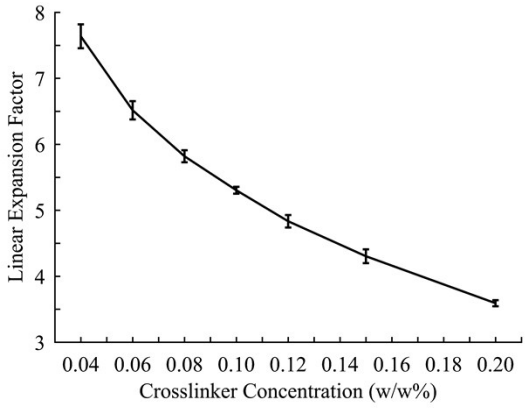

Fig. S2 Expansion capacity of sample-free hydrogels with different crosslinker concentrations (mean ± SD, n = 3 biological replicates). For each crosslinker concentration, we synthesized three sample-free hydrogels, and measured the diameter of the gels before and after expansion. Mean value and standard deviation were calculated with the three hydrogels.

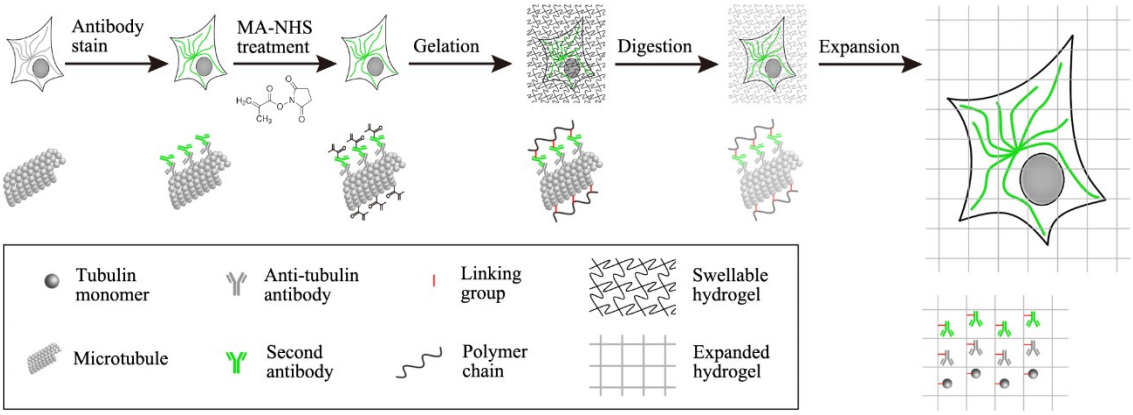

Fig. S3 Illustration of expansion microscopy (this illustration is inspired by protein-retention expansion microscopy).[4]

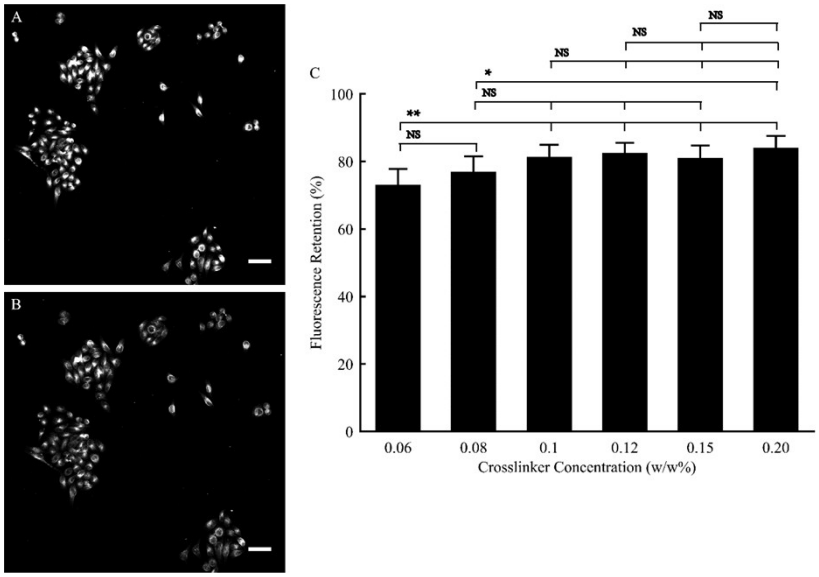

Fig. S4 Fluorescence retention of immunostained microtubule after digestion processing. (A) Pre-digestion image. (B) Post-digestion image. Both scale bars are 100 μm. (C) Bar graph of estimated fluorescence retention caused by digestion processing for difference crosslinker concentrations (mean ± SD, n = 4 biological replicates). NS: not significance, *P<0.05, **P<0.01, Welch's ANOVA. For each column, we immunostained four dishes of HeLa cells and carry out processing. We selected one region of each sample for fluorescence retention analysis. Mean value and standard deviation were calculated with the four regions.

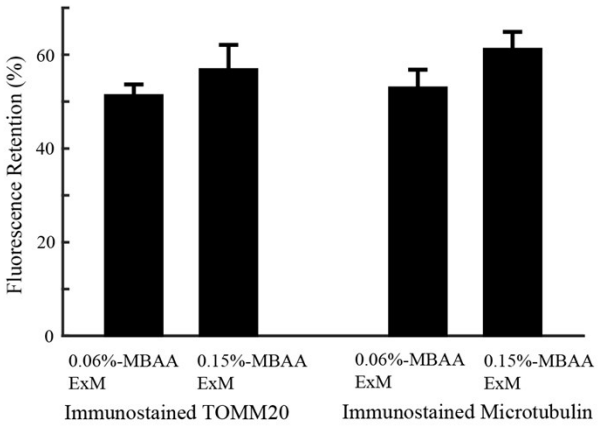

Fig. S5 Fluorescence retention of 0.06%-MBAA ExM and 0.15%-MBAA ExM (conventional ExM) before-after digestion and expansion (mean ± SD, n = 3 biological replicates). Two-way ANOVA shows that the differences in fluorescence retention levels at MBAA concentration factor were statistically significant ($P < 0.05$), and there was no significant difference in fluorescence retention levels at target protein factor. For microtubule fluorescence retention, HeLa cells was immunostained with anti-tubulin antibody and Alexa Fluor 488 labeled second antibody. For TOMM20 (a mitochondrial membrane protein) fluorescence retention, HeLa cells was immunostained with anti-TOMM20 antibody and Alexa Fluor 488 labeled second antibody. Cell regions were selected with image segmentation and integrated density of cell regions were measured in ImageJ. Fluorescence retention was estimated with the measurement before-after digestion and expansion images. For each column, we immunostained three dishes of HeLa cells and carry out processing. We selected one region of each sample for fluorescence retention analysis. Mean value and standard deviation were calculated with the three regions.

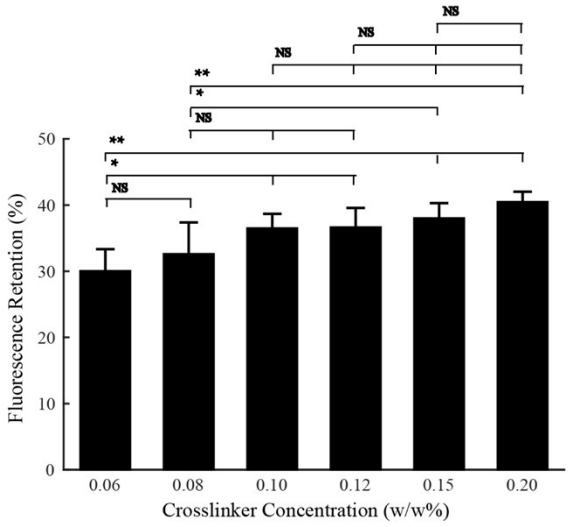

Fig. S6 Fluorescence retention of immunostained microtubule before-after ExM processing for difference crosslinker concentrations (mean ± SD, n = 3 biological replicates). NS: not significance, *P<0.05, **P<0.01, Welch's ANOVA. HeLa cells were immunostained with anti-tubulin antibody and Alexa Fluor 488 labeled second antibody. After ExM processing, samples were immersed in digestion buffer for two times and imaged. Corresponding cell regions were selected with polygon selection and integrated density of the regions were measured in ImageJ. Fluorescence retention was estimated with the measurement before-after ExM processing images. For each column, we immunostained three dishes of HeLa cells and carry out ExM processing. We selected one region of each sample for fluorescence retention analysis. Mean value and standard deviation were calculated with the three regions.

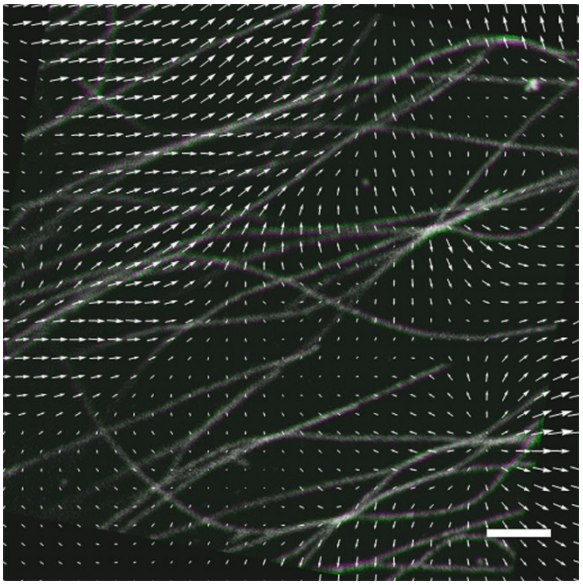

Fig. S7 Overlay of post-expansion image before (magenta) and after (green) B-spline registration processing which deforms the post-expansion image to fit the pre-expansion image. The image was acquired with 0.06%-ExM and performed similarity transformation and B-spline transformation in elastix with the method descripted by Jo. Vaughan's group.[2,3] Deformation vector field were acquired with a Mathematica script written by them.[3] Arrows indicate deformation vectors to align the post-expansion image to the pre-expansion image. Scale bar is 1 μm

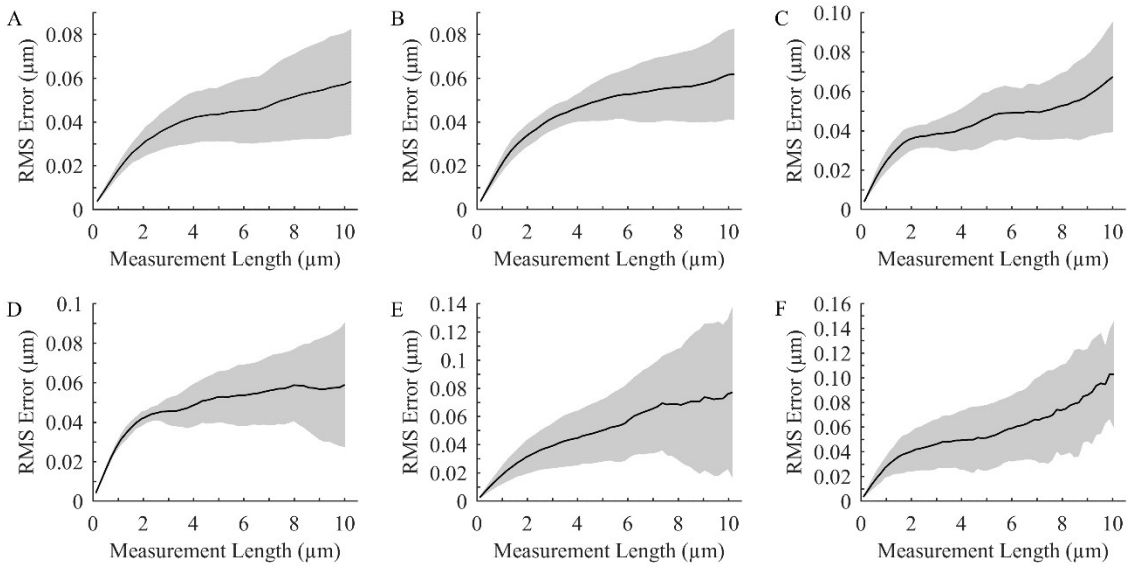

Fig. S8 RMS error analysis of ExM with different crosslinker concentrations. Microtubule was immunostained with anti-tubulin antibody and Alexa Fluor 488 labeled second antibody and used for RMS error analysis. From A to F, the MBAA concentration successively is 0.20%, 0.15%, 0.12%, 0.10%, 0.08% and 0.06%. Black line represents mean value and shaded area represents standard deviation (n = 3 biological replicates). For each picture, we immunostained three dishes of HeLa cells and carry out ExM processing. We selected one region of each sample for RMS error analysis. Mean value and standard deviation were calculated with the three regions.

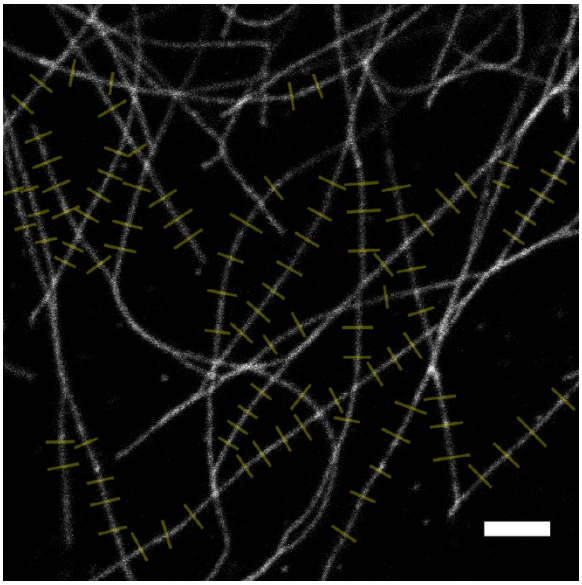

Fig. S9 Cross-sectional profile analysis for microtubule. Semitransparent yellow lines perpendicular to microtubule orientation were used for FWHM measurement. Scale bar is 1 μm.

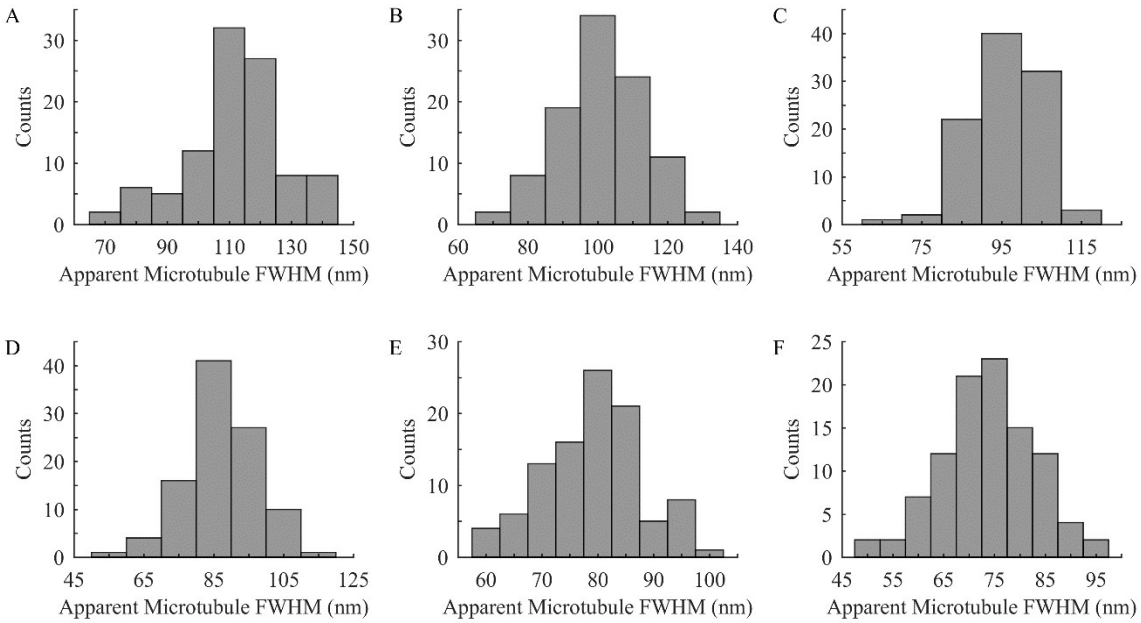

Fig. S10 Microtubule FWHM analysis of ExM with different crosslinker concentrations. From A to F, the MBAA concentration successively is 0.20%, 0.15%, 0.12%, 0.10%, 0.08% and 0.06%.

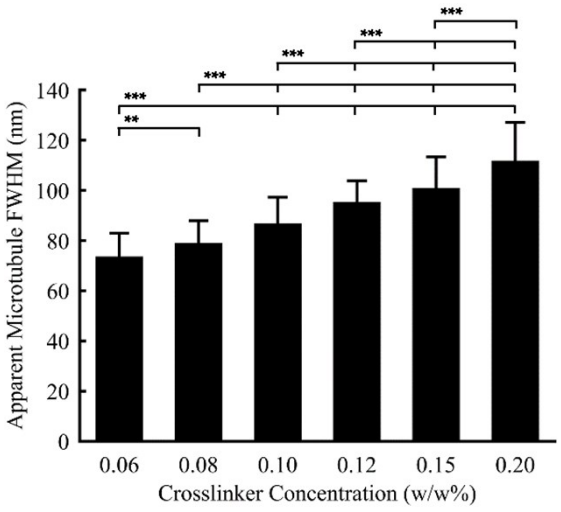

Fig. S11 Bar graph of apparent microtubule FWHM measured by ExM with different crosslinker concentrations (mean ± SD, n = 100 microtubule profiles). **P<0.01, ***P<0.001, Welch's ANOVA.

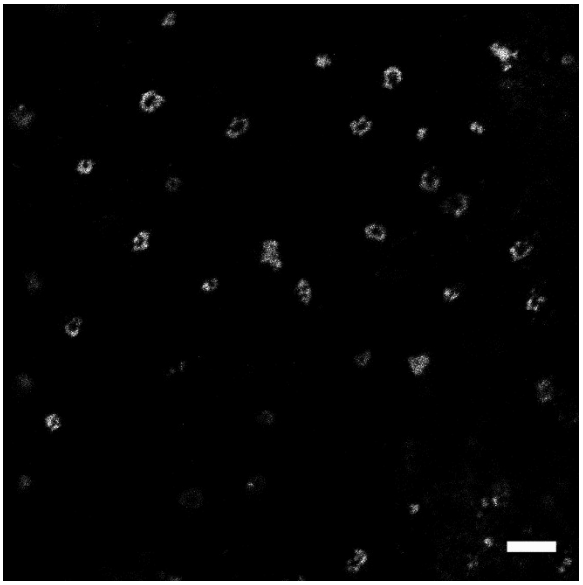

Fig. S12 Clathrin-coated pits (CCPs) image obtained with 0.06%-MBAA ExM. Scale bar is 1 μm.

Supplementary Table

Table S1 Problems and solutions

Problems	Solutions
Cannot find a cell in post-expansion sample.	The cells maybe out of focal distance. If fix sample on confocal dish with agarose, don't let the agarose solution get into the space between the glass bottom and the hydrogel.
Microtubules are not clear enough in pre-expansion sample.	Extract cells with PEM buffer containing 0.5% Triton X-100.
Gel solution cannot gelatinize in the well.	Isolate the gel solution from air with plastic wrap.
Capture the same cell in pre- and post-expansion sample.	Use a pre-expansion image acquired with 10× objective as a 'map'.
Post-expansion show serious distortions.	Do not pressure the hydrogel, when it is expanding in water.
Expanded microtubules appear a lot of breakpoint.	Increase digestion time.

References

1 J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld and B. Schmid, *Nat. Methods*, 2012, **9**, 676.

2 S. Klein, M. Staring, K. Murphy, M. A. Viergever and J. P. Pluim, *IEEE Trans. Med. Imaging*, 2009, **29**, 196-205.

3 T. J. Chozinski, A. R. Halpern, H. Okawa, H.-J. Kim, G. J. Tremel, R. O. Wong and J. C. Vaughan, *Nat. Methods*, 2016, **13**, 485-488.

4 P. W. Tillberg, F. Chen, K. D. Piatkevich, Y. Zhao, C.-C. J. Yu, B. P. English, L. Gao, A. Martorell, H.-J. Suk and F. Yoshida, *Nat. Biotechnol.*, 2016, **34**, 987–992.