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Supplementary Materials for

Expansion microscopy with ninefold swelling (NIFS) hydrogel permits cellular ultrastructure imaging on conventional microscope

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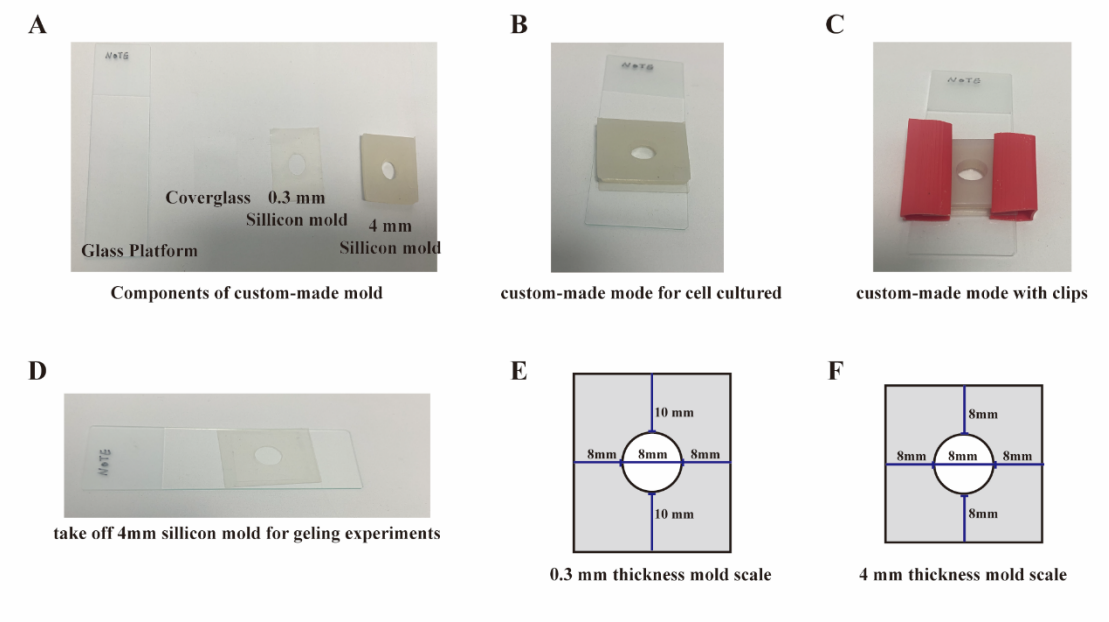

Figure S1. The custom-made mold used in expansion microscopy. (A) graph is all components of home-made mold. Glass platform for protection fragile coverglass. Coverglass was used for cell growth and imaging. 0.3mm thickness silicon mold applied to gelation process. 4mm thickness silicon mold used for the cell culture medium. (B) Assembled home-made mold and with clips (C) guarantees the medium leakage at the cell growth time. (D) custom-made mold except 4mm thickness silicon was used for later gelation experiments. The specific silicon mold parameters of 0.3 mm thickness (E) and 4mm (F).

In order to remove 4 mm thickness silicon easily, the size would be a little bit smaller than that in 0.3 mm thickness silicon.

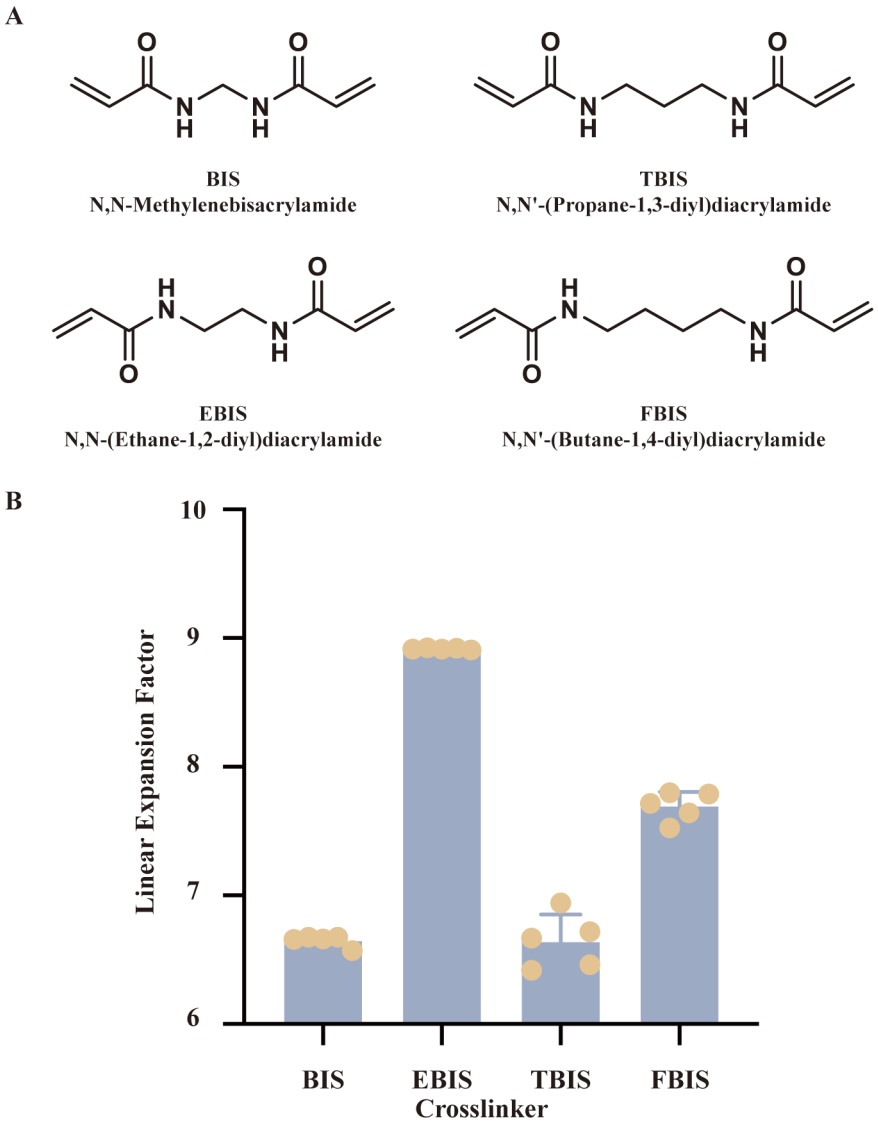

Figure S2: Linear expansion capacity of different length crosslinkers. (A) Structures of different length crosslinkers. (B) Linear expansion capacity of sample-free gel with different crosslinker length. Error bars represent standard deviation, n = 5. All crosslinkers' concentration is 0.06% (w/w).

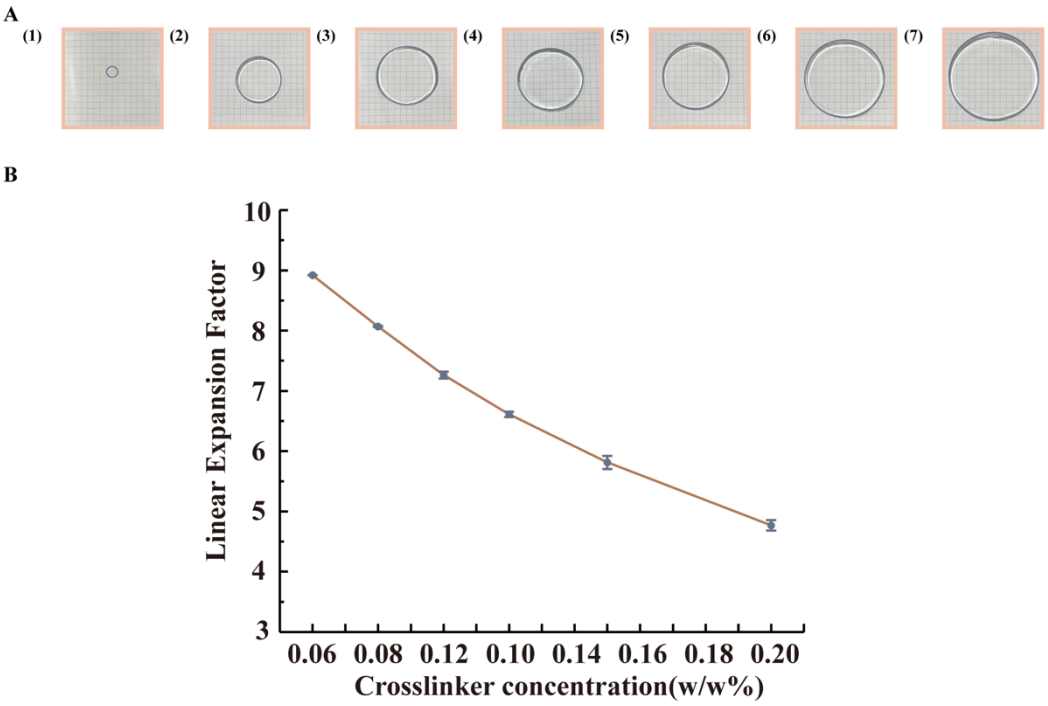

Figure S3: Linear expansion capacity of sample-free gel with different crosslinker concentration. (A) photographs of hydrogels with (1) pre-expansion, (2) 0.20% (w/w) crosslinker, (3) 0.15% (w/w) crosslinker, (4) 0.12% (w/w) crosslinker, (5) 0.10% (w/w) crosslinker, (6) 0.08% (w/w) crosslinker, (7) 0.06% (w/w) crosslinker. (B) Statics different concentration analysis of linear expansion factor between pre-expansion and post-expansion. Error bars represent SD, n = 5.

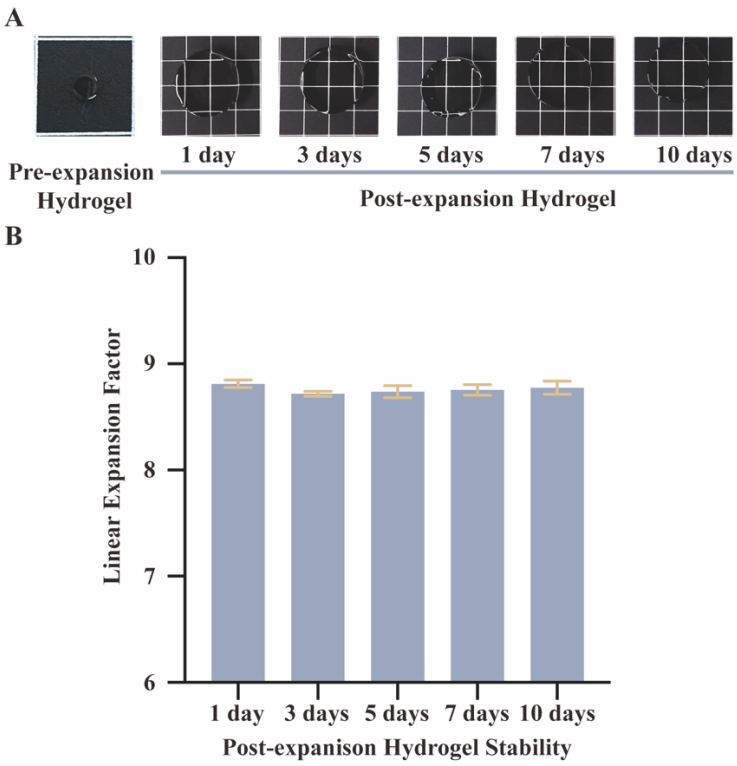

Figure S4. The stability of post-expansion hydrogel with 0.06% (w/w) crosslinker concentration. (A) Photographs of pre-expansion hydrogel and post-expansion hydrogels (1 day, 3 days, 5 days, 7 days, and 10 days). (B)Linear expansion factor of post-expansion hydrogel at different days. Error bars represent SD, n=3.

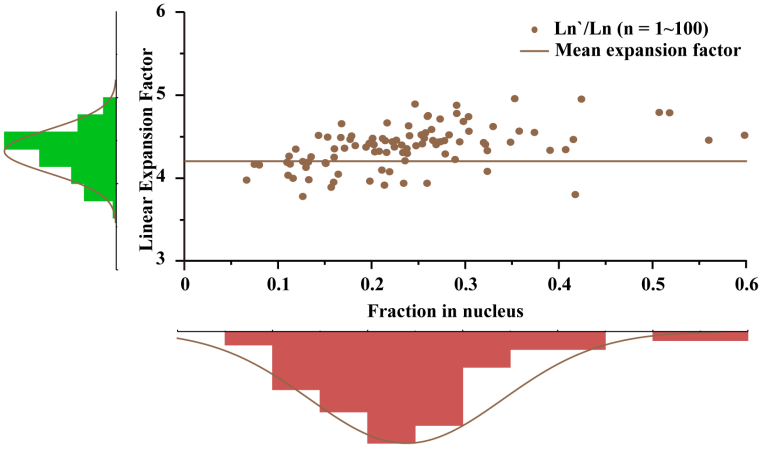

Figure S5. The expansion factor (0.20% (w/w) EBIS concentration) of nucleus pairwise ratio in pre-and post-ExM process versus the fraction of nucleus (brown line, mean expansion factor, Mean ± SD = 4.38 ± 0.253)

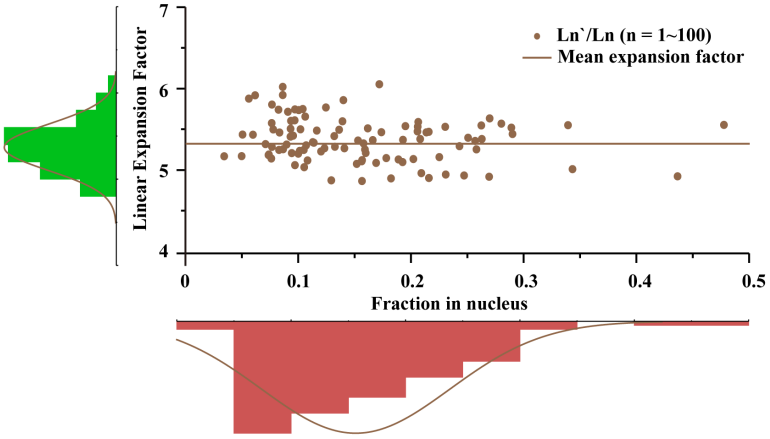

Figure S6. The expansion factor (0.15% (w/w) EBIS concentration) of nucleus pairwise ratio in pre-and post-ExM process versus the fraction of nucleus (brown line, mean expansion factor, Mean ± SD = 5.37 ± 0.269)

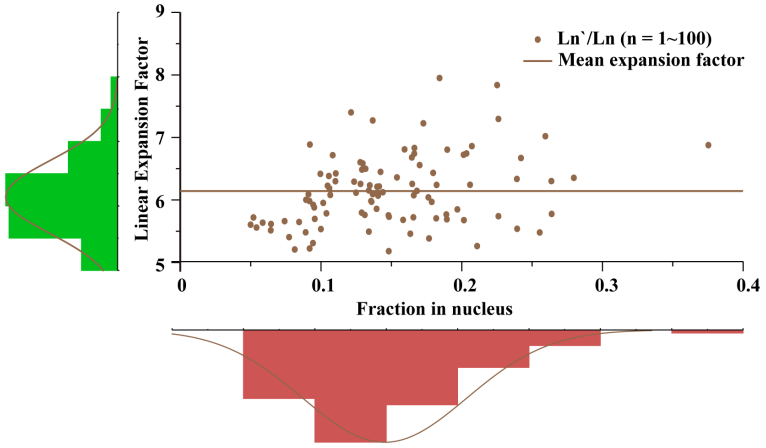

Figure S7. The expansion factor (0.12% (w/w) EBIS concentration) of nucleus pairwise ratio in pre-and post-ExM process versus the fraction of nucleus (brown line, mean expansion factor, Mean ± SD = 6.14 ± 0.563)

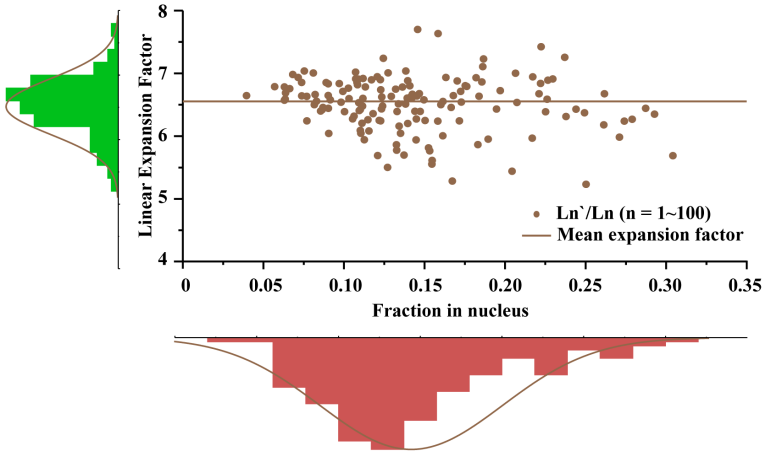

Figure S8. The expansion factor (0.10% (w/w) EBIS concentration) of nucleus pairwise ratio in pre-and post-ExM process versus the fraction of nucleus (brown line, mean expansion factor, Mean ± SD = 6.51 ± 0.430)

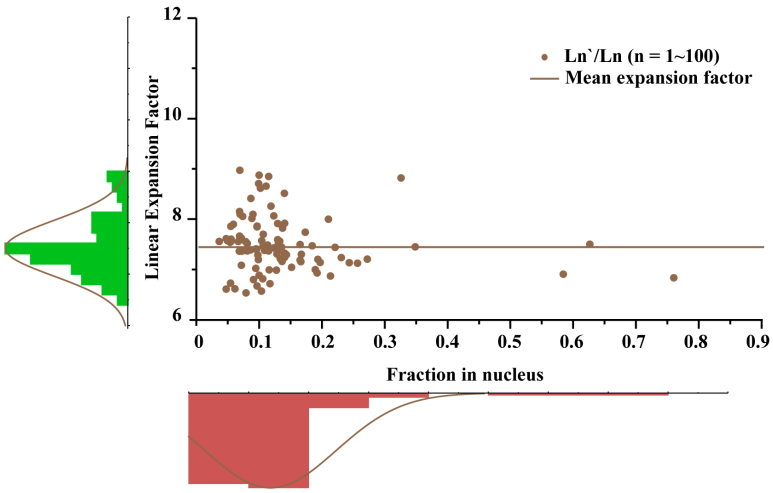

Figure S9. The expansion factor (0.08% (w/w) EBIS concentration) of nucleus pairwise ratio in pre-and post-ExM process versus the fraction of nucleus (brown line, mean expansion factor, Mean ± SD = 7.50 ± 0.538)

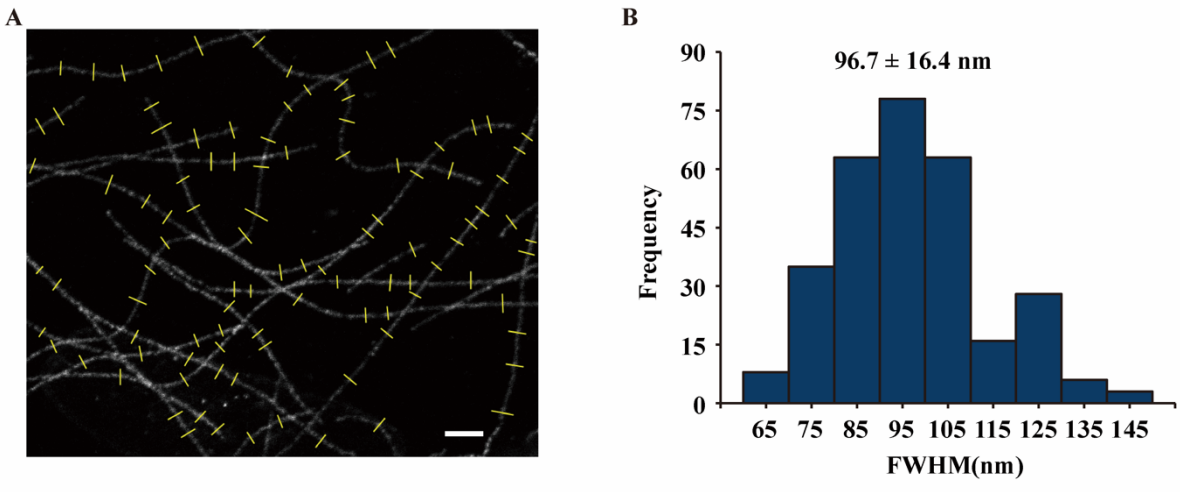

Figure S10. The 0.20 % (w/w) EBIS of NIFS hydrogel in expansion microscopy. The left picture to get the intensity profiles of a line profile perpendicular to microtubules orientation. The right picture was the histogram of full width at half maximum (FWHM) after Gaussian fitted intensity profile. Scale bar: 1 μm.

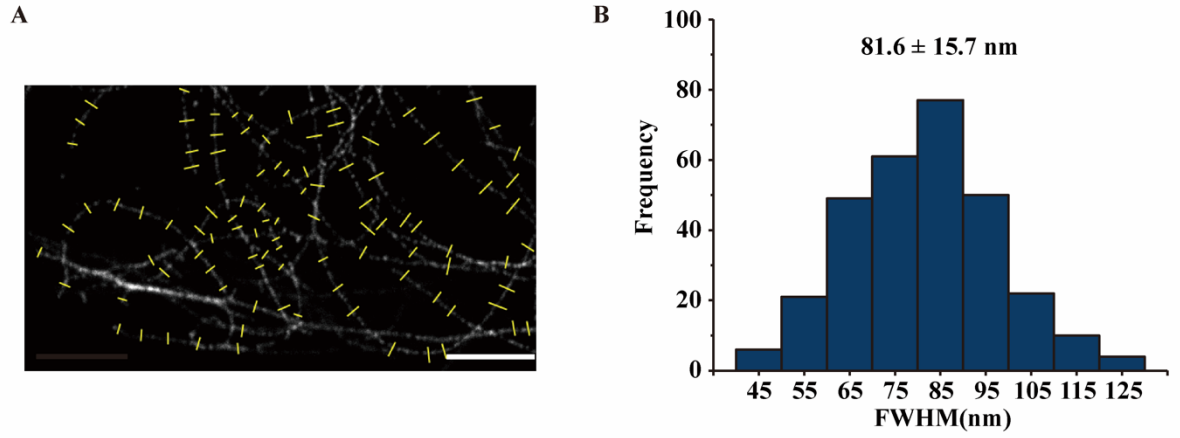

Figure S11. The 0.15 % (w/w) EBIS of NIFS hydrogel in expansion microscopy. The left picture to get the intensity profiles of a line profile perpendicular to microtubules orientation. The right picture was the histogram of full width at half maximum (FWHM) after Gaussian fitted intensity profile. Scale bar: 2 μm.

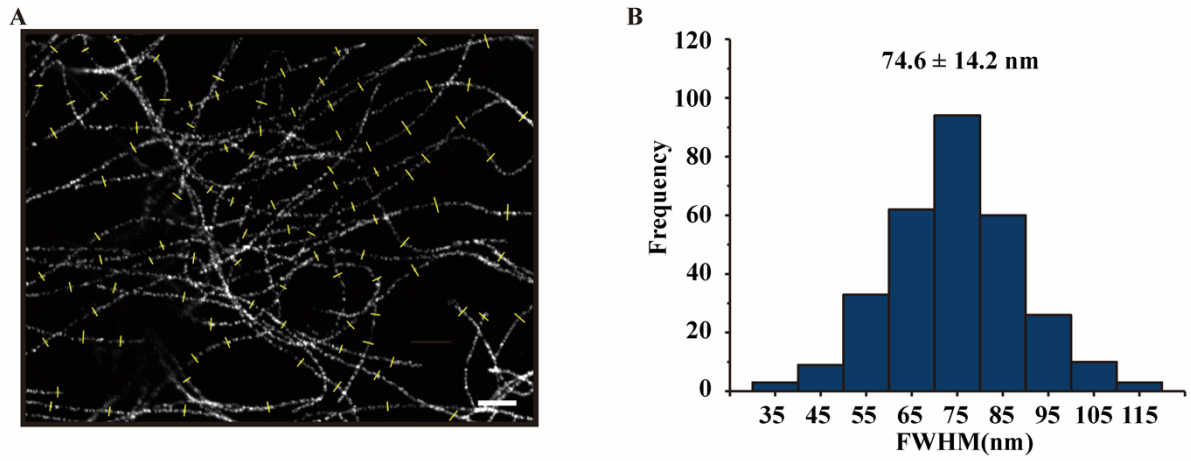

Figure S12. The 0.12 % (w/w) EBIS of NIFS hydrogel in expansion microscopy. The left picture to get the intensity profiles of a line profile perpendicular to microtubules orientation. The right picture was the histogram of full width at half maximum (FWHM) after Gaussian fitted intensity profile. Scale bar: 1 μm.

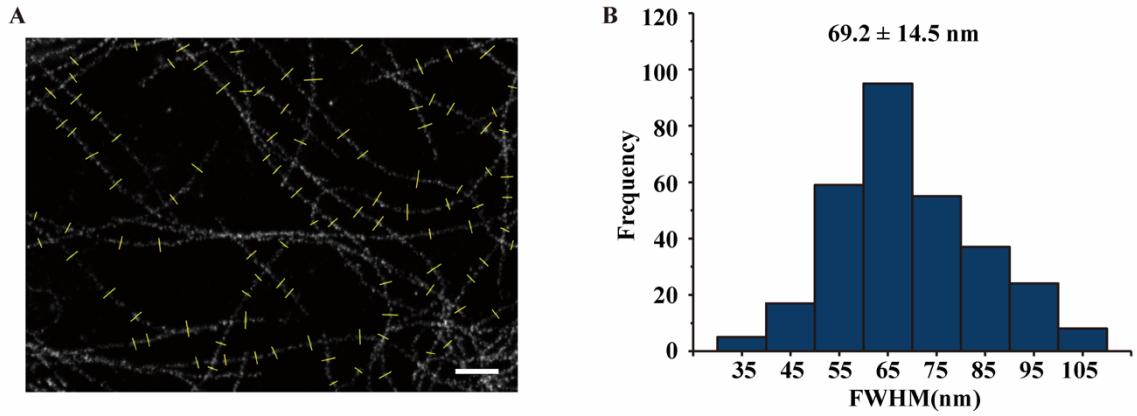

Figure S13. The 0.10 % (w/w) EBIS of NIFS hydrogel in expansion microscopy. The left picture to get the intensity profiles of a line profile perpendicular to microtubules orientation. The right picture was the histogram of full width at half maximum (FWHM) after Gaussian fitted intensity profile. Scale bar: 1 μm.

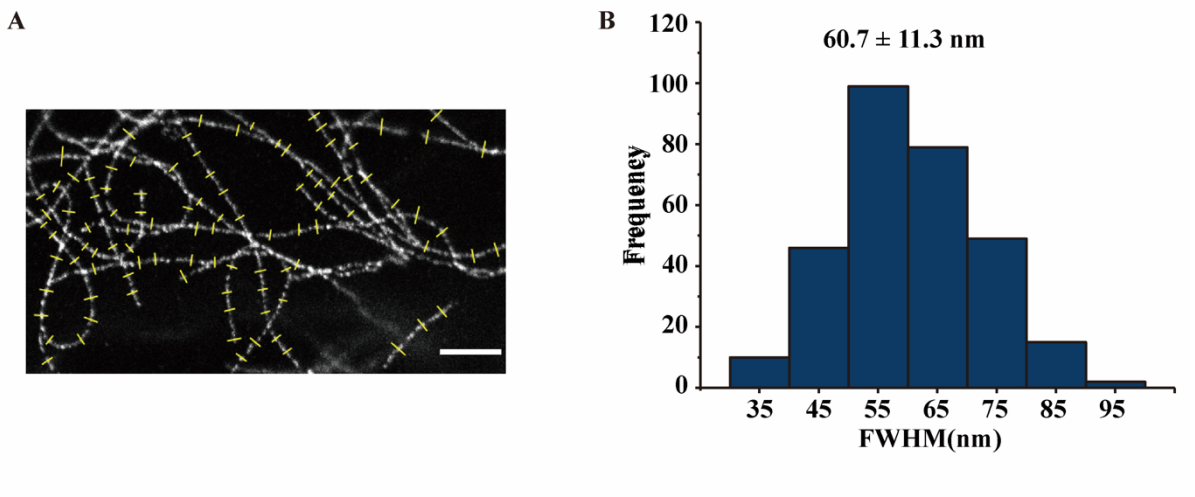

Figure S14. The 0.08 % (w/w) EBIS of NIFS hydrogel in expansion microscopy. The left picture to get the intensity profiles of a line profile perpendicular to microtubules orientation. The right picture was the histogram of full width at half maximum (FWHM) after Gaussian fitted intensity profile. Scale bar: 1 μm.

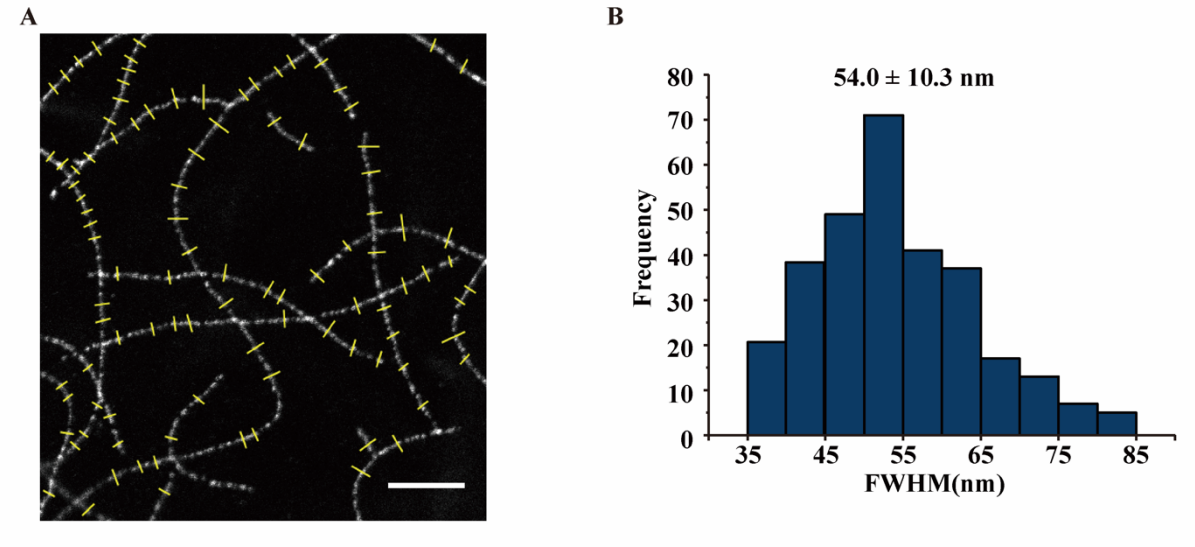

Figure S15. The 0.06 % (w/w) EBIS of NIFS hydrogel in expansion microscopy. The left picture to get the intensity profiles of a line profile perpendicular to microtubules orientation. The right picture was the histogram of full width at half maximum (FWHM) after Gaussian fitted intensity profile. Scale bar: 1 μm.

Expansion factor		Crosslinker concentration (% (w/w))					
		0.06	0.08	0.10	0.12	0.15	0.20
Cell-embedded hydrogels	Macroscopic scale	8.12 ± 0.131	7.48 ± 0.152	6.70 ± 0.099	6.04 ± 0.106	5.45 ± 0.118	4.58 ± 0.090
	Microscopic scale	8.21 ± 0.693	7.50 ± 0.538	6.51 ± 0.430	6.14 ± 0.563	5.37 ± 0.269	4.38 ± 0.253
	Nucleus registration	8.19 ± 0.038	7.29 ± 0.017	6.42 ± 0.059	6.08 ± 0.078	5.24 ± 0.018	4.45 ± 0.023
	Nanoscopic scale	8.27 ± 0.122	7.51 ± 0.097	6.72 ± 0.126	6.15 ± 0.092	5.47 ± 0.084	4.37 ± 0.036
Cell-free hydrogel		8.92 ± 0.006	8.07 ± 0.008	7.28 ± 0.050	6.61 ± 0.032	5.80 ± 0.083	4.74 ± 0.070

Figure S16. The summary of expansion ability for EBIS crosslinker hydrogel. Macroscopic scale, Mean ± SD, n = 5 biological replicates, microscopic scale, Mean ± SD, n = 100 from 3 biological replicates, nucleus registration method, Mean ± SD, n = 3 biological replicates, nanoscopic scale, Mean ± SD, n = 5 biological replicates, each replicate chooses one region, cell-free hydrogel, Mean ± SD, n = 5.

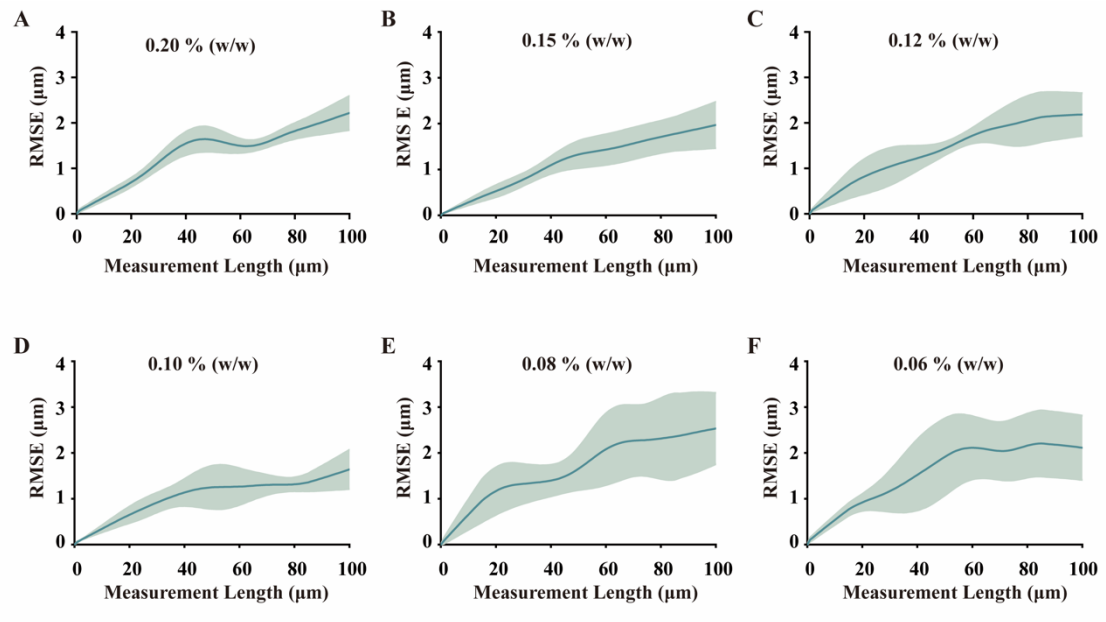

Figure S17: Distortion analysis of cell nucleus with different EBIS crosslinker concentrations. A. 0.20% (w/w) crosslinker, B. 0.15% (w/w) crosslinker, C. 0.12% (w/w) crosslinker, D. 0.10% (w/w) crosslinker, E. 0.08% (w/w) crosslinker, F. 0.06% (w/w) crosslinker. Solid line represents the mean value of RMSE and shaded area represents standard deviation (n = 3 biological samples, we selected one zone each sample for RMSE analysis).

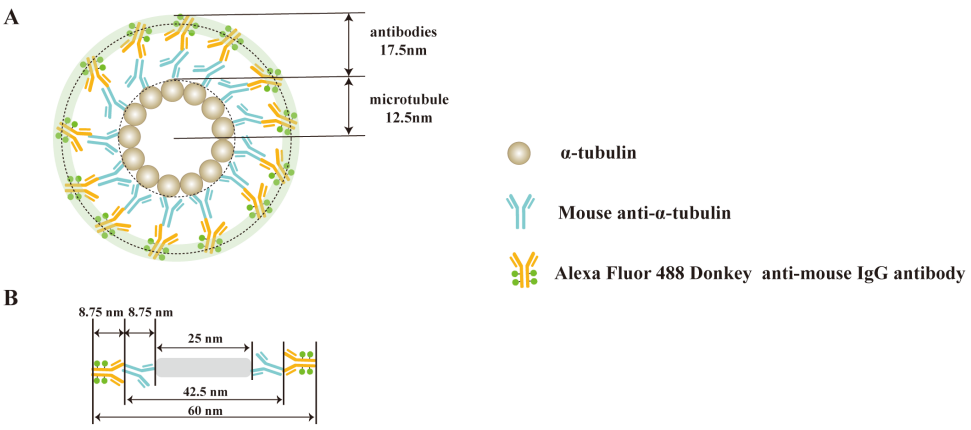

Figure S18. The size distribution of microtubule complex with antibody immunostaining.

(A) The 2D equatorial view. (B) The 2D top view

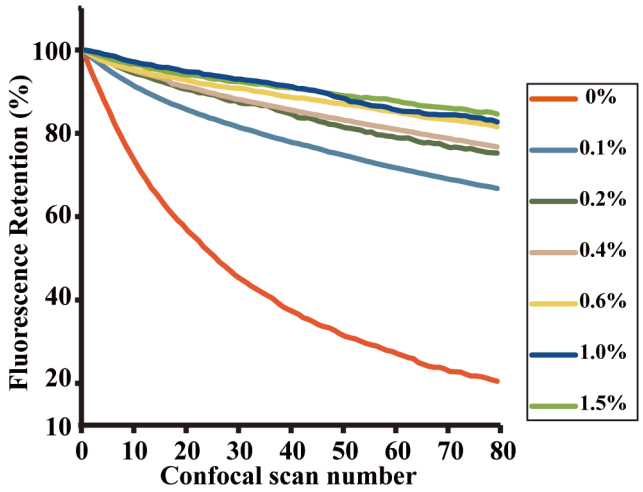

Figure S19. The optimized concentration of 1,4-Diazabicyclo [2.2.2] octane (DABCO) in water (0-1.5% (v/v)) facilitates over 90% fluorescence retention during long-term exposure. Confocal laser consecutive scans the same region in fluorescent cell dishes (Fluorescence: Alexa Fluor 488, laser: 488, laser power: 10% of 6.7mW lens: 10x/0.3NA).

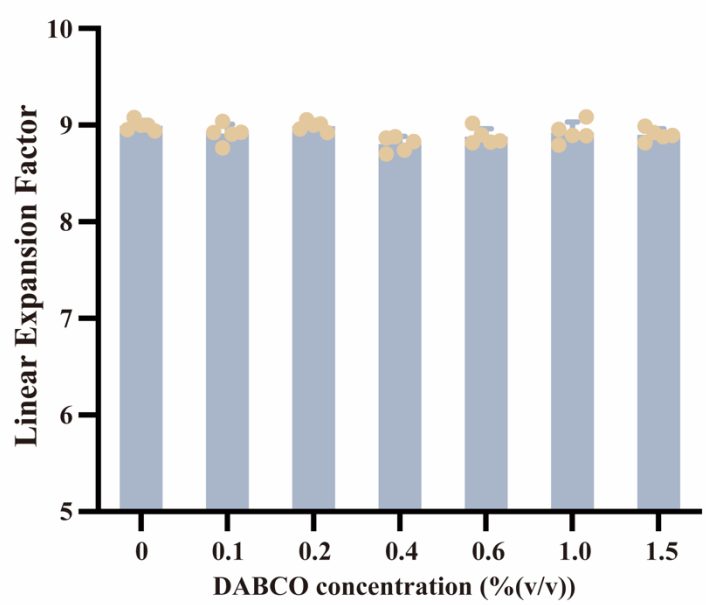

Figure S20. Linear expansion capacity of sample free hydrogel with different DABCO concentrations (from 0-1.5% (v/v)). Error bars represent SD, n=5.

In conventional microscopy, the resolution estimation method was determined by the formula attached below:

$$\sigma=\lambda/NA$$

where σ represents resolution, λ represents wavelength, and NA represents Numerical Aperture.

A typical confocal microscopy (63x/1.40NA oil-immersion lens) permits a resolution of 240 nm. For the NIFS hydrogel ExM method, the size of sample-embedded NIFS hydrogel expands 8.27-fold than itself in one dimension. Therefore, the theoretical resolution is 240/8.27 = 29 nm at the same machine setup.

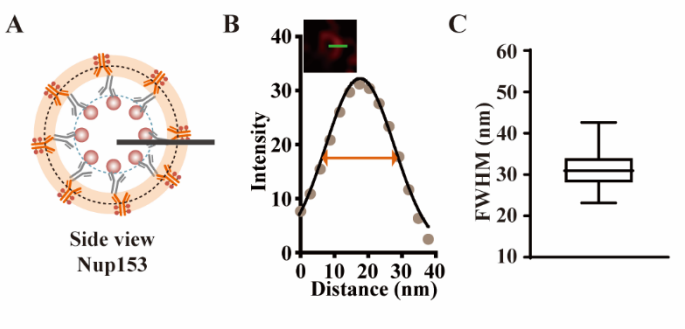

Figure S21. Estimate resolution of NIFS hydrogel expansion microscopy. (A) The side view of Nup153 structure with antibody tagged. The black line indicates the cross-section that we adopted for FWHM calculation. (B) The inset image indicates a single Nup153 circle, and the green line also indicates the cross-section we adopted for FWHM calculation. The corresponding fluorescence intensity along this green line is further recorded underneath as a function of distance from the circle center (brown dots), while the Gaussian curve fitting is also generated based on these brown dots to estimate FWHM. (C) The average of FWHM for Nup153 (n = 100 from 3 replicates, each replicate chooses one region) and the range distribution.

To estimate the resolution of NIFS hydrogel in ExM, the FWHM of Nup153 circles was used. The theoretical Nup153 circular structure is displayed in figure S18A, where the horizontal black line indicates the cross-section that we adopted for FWHM calculation. In figure S18B, the inset image indicates a single Nup153 circle, and the green line also indicates the cross-section we adopted for FWHM calculation. The corresponding fluorescence intensity along this green line is further recorded underneath as a function of distance from the circle

center (brown dots), while the Gaussian curve fitting is also generated based on these brown dots to estimate FWHM. Our data indicate that the FWHM is 31.5 ± 4.3 nm (n=100) (figure S18C). This estimated resolution is therefore consistent with the theoretical calculation of microscopy (63×1.4NA oil lens) through the expansion process (~8.24x) as indicated above.

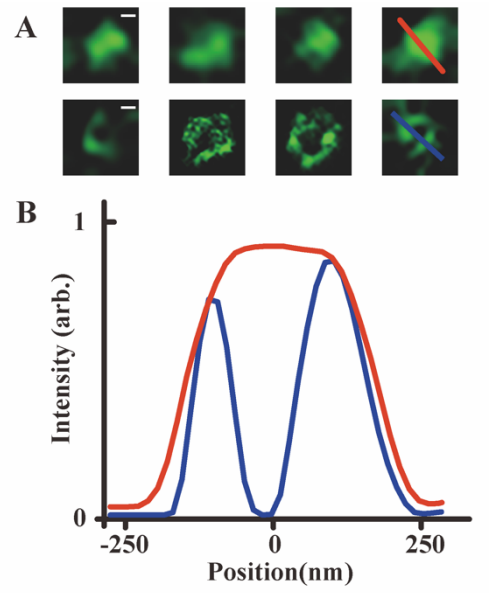

Figure S22. Clathrin-Coated pit (CCP) analysis in ExM with NIFS hydrogel. (A). Images of pre- and post-expansion status. (B) The corresponding line profiles of pre-expansion (red line) and post-expansion (blue line). Scale bar: 200nm. All scales are pre-expansion dimension.

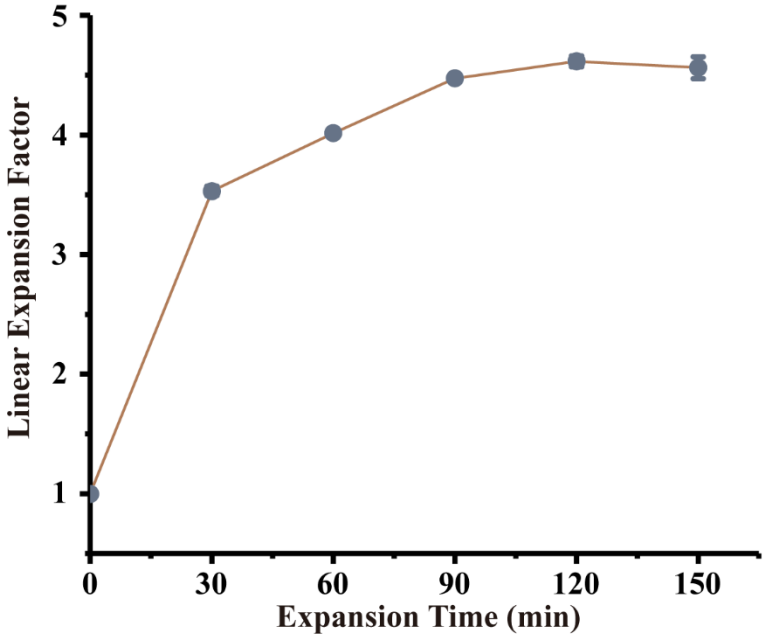

Figure S23. The optimum time of expansion process. change water every 30 min. (mean± SD, n=three biological replicates). We synthesized three replicated hydrogels and measured the diameter of gels before change the expansion buffer, every 30min change once. Error bars represent SD., n = 3.

Table S1. Sample preparation and imaging conditions.

Figure	Sample name	fixation	antibodies	Imaging Condition	Notes
Fig.2E	Pre-Expansion	3.2% PFA in 1xPBS for 10min	Hoechst (5ug/ml 30min)	EC plnN 25x/ 0.3NA Laser: 405(5%)	
	Post-Expansion	3.2%PFA in 1xPBS for 10min	Hoechst (2ug/ml 30min)	EC plnN 10x/ 0.3NA Laser: 405(6.5%)	19 Slice MIP
Fig.2H	Pre-Expansion	PEM with triton extract for 30s 3.2%PFA+0.1% GA in PEM for 10min	Mouse anti a-Tubulin (30304ES40) 1:100 AF488 goat-anti mouse IgG (H+L) (33206ES60) 1:300	Pln Apo 63x/1.4NA Oil Laser: 488(0.14%)	
	Post-Expansion	PEM with triton extract for 30s 3.2%PFA+0.1% GA in PEM for 10min		Pln Apo 25x/0.8NA Oil Laser: 488(20%)	
Fig.3B	Pre-Expansion	PEM with triton extract for 30s 3.2%PFA+0.1% GA in PEM for 10min	Mouse anti a-Tubulin (30304ES40) 1:100 AF488 goat-anti mouse IgG (H+L) (33206ES60) 1:300	Pln Apo 63x/1.4NA Oil Laser:488(0.14%)	
	Post-Expansion	PEM with triton extract for 30s 3.2%PFA+0.1% GA in PEM for 10min		Pln Apo 25x/0.8NA Oil Laser:488(20%)	
Fig.3G	Microtubules	1x PEM with 0.1% Triton X-100 extract for 30s 3.2%PFA+0.1% GA in 1x PEM for 10min	Mouse anti a-Tubulin (30304ES40) 1:100 AF594 goat-anti mouse IgG (H+L) (34112ES60) 1:300	Pln Apo 63x/1.4NA Oil Laser: 561(20%)	
Fig.3L	Pre-Expansion	3.2% PFA in 1x PBS for 10min	Hoechst (5ug/ml 30min)	EC plnN 25x/ 0.8NA Laser:405(5%)	
	Post-Expansion	3.2% PFA in 1x PBS	Hoechst	EC plnN 10x/ 0.3NA	8slice

		for 10min	(2ug/ml 30min)	Laser:405(8%)	MIP
Fig.4B	Upper view	3.2% PFA in 1x PBS for 10min	Mouse anti-Nup153[QE5] (ab24700) 1:150 AF594 goat-anti mouse IgG (H+L) (34112ES60) 1:300 Hoechst (5ug/ml 30min)	Pln Apo 63x/1.4NA Oil Laser: 405(0.06%) 561(0.6%)	8 Slice (5.6μm) Slice 2
	Central				Slice 5
	Downer view				Slice 7
	Different y stack				Slice 5
Fig.4C	Nup153	3.2% PFA in 1x PBS for 10min		Pln Apo 63x/1.4NA Oil Laser:561(45%)	Z stack z-04 for radii analysis 4
Fig.4E	Post-expansion Nup153(3D)	3.2% PFA in 1x PBS for 10min		Pln Apo 63x/01.40NA Oil Laser: 561(15%)	4 tiles 46 Slices (45μm)
Fig.4G	Nup153(2D equator)	3.2% PFA in 1x PBS for 10min		Pln Apo 63x/1.4NA Oil Laser: 561(15%)	Slice 24
Fig.S10A	Tubulins (0.20%)	1x PEM with 0.1% Triton X-100 extract for 30s 3.2%PFA+0.1% GA in 1x PEM for 10min	Mouse anti a-Tubulin (30304ES40) 1:100 AF488 goat-anti mouse IgG (H+L) (33206ES60) 1:300	Pln Apo 63x/1.4NA Oil Laser: 488 (12%)	
Fig.S11A	Tubulins (0.15%)			Pln Apo 63x/1.4NA Oil Laser: 488 (12%)	
Fig.S12A	Tubulins (0.12%)			Pln Apo 63x/1.4NA Oil Laser: 488 (12%)	
Fig.S13A	Tubulins (0.10%)			Pln Apo 63x/1.4NA Oil Laser: 488 (15%)	
Fig.S14A	Tubulins (0.08%)			Pln Apo 63x/1.4NA Oil Laser: 488 (18%)	
Fig.S15A	Tubulins (0.06%)			Pln Apo 63x/1.4NA Oil Laser: 488 (20%)	
Fig.S22A	Clathrin-Coated pit(pre-expanison)	3.2% PFA in 1x PBS for 10min	Mouse monoclonal to clathrin heavy chain(ab172958) 1:150 AF488 goat-anti mouse IgG (H+L) (33206ES60) 1:300	Pln Apo 63x/1.4NA Oil Laser: 488 (0.8%)	
	Clathrin-Coated pit(post-expanison)			Pln Apo 63x/1.4NA Oil Laser: 488 (12%)	

MIP: Maximum Intensity Projection analysis