Supplementary Table 1. List of chemicals

Product Name	Vendor	Product number
Gelation		
Sodium acrylate	Sigma	408220
Acrylamide	Sigma	A9099
N,N′-Methylenebisacrylamide (BIS)	Sigma	M7279
Ammonium Persulfate (APS)	Sigma	A3678
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma	T7024
4-Hydroxy-TEMPO (H-tempo)	Sigma	176141
N,N'-(1,2-Dihydroxyethylene)bisacrylamide (DHEBA)	Tokyo Chemical Industry	D2864
N,N'-Cystaminebisacrylamide (BAC)	Polysciences	09809
Tris(2-carboxyethyl)phosphine (TCEP)	Sigma	646547
Cell and neuron culture		
Eagle's Minimum Essential Medium (EMEM)	American Type Culture Collection	30-2003
Fetal Bovine Serum	Corning	35-010-CV
Penicillin-Streptomycin Solution	Corning	30-002-CI
Fixation and staining		
Paraformaldehyde	Electron Microscopy Sciences	15710
Glutaraldehyde	Electron Microscopy Sciences	16020
Triton X-100	Sigma	X100
Glycine	Sigma	50046
PBS 10x	Life Technologies	70011-044
Dextran Sulfate 50%	Millipore	S4030
SSC 20x	Life Technologies	15557
Yeast tRNA	Roche	10109495001
Normal Donkey Serum	Jackson ImmunoResearch	017-000-001
1,4-Piperazinediethanesulfonic acid (PIPES)	Sigma	P1851
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)	Sigma	E38889
Magnesium chloride	Sigma	M8266
Digestion		
Proteinase K	New England Biolabs	P8107S
Ethylenediaminetetraacetic acid	Sigma	EDS
Guanidine HCl	Sigma	G3272
Tris-HCl, 1M pH 8.0	Life Technologies	AM9855
STORM imaging		
β-Mercaptoethanol	Sigma	M3148
Glucose oxidase	Sigma	G2133
Catalase	Sigma	C100
Glucose	Sigma	G7528

Supplementary Table 2. DNA with a 5’amine modification

Name	Sequence	Modifications
A1’ 5’amine	AA CCG AAT ACA AAG CAT CAA CG	5’Amine
A2’ 5’amine	AA GGT GAC AGG CAT CTC AAT CT	5’Amine
A3’ 5’amine	AA GTC CCT GCC TCT ATA TCT CC	5’Amine
B1’ 5’amine	AA TAC GCC CTA AGA ATC CGA AC	5’Amine
C1’ 5’amine	AA GAC CCT AAG CAT ACA TCG TC	5’Amine

Supplementary Table 3. DNA bearing a gel-anchoring moiety

Name	Sequence	Modifications
A1 5’acrydite 3’alexa488	CG TTG ATG CTT TGT ATT CGG T	5’Acrydite 3’Alexa488
A2 5’acrydite	AG ATT GAG ATG CCT GTC ACC	5’Acrydite
A3 5’acrydite	GG AGA TAT AGA GGC AGG GAC	5’Acrydite
B1 5’acrydite	GT TCG GAT TCT TAG GGC GTA	5’Acrydite
C1 5’acrydite	GA CGA TGT ATG CTT AGG GTC	5’Acrydite

Supplementary Table 4. DNA for 2nd expansion (no signal amplification)

Name	Sequence	Modifications
A1’ 5’acrydite 3’atto565	CCG AAT ACA AAG CAT CAA CG	5’Acrydite 3’Atto565
C1’ 5’acrydite 3’alexa488	GAC CCT AAG CAT ACA TCG TC	5’Acrydite 3’Alexa488

Supplementary Table 5. Linker DNA for DNA hybridization-based signal amplification (see Supplementary Fig. 10)

Name	Sequence	Modifications
A3’ 4A1’ 5’acrydite	GT CCC TGC CTC TAT ATC TCC ATA CCG AAT ACA AAG CAT CAA TAC CGA ATA CAA AGC ATC AAT ACC GAA TAC AAA GCA TCA ATA CCG AAT ACA AAG CAT CA	5’Acrydite
B1’ 4B2’ 5’acrydite	AT ACG CCC TAA GAA TCC GAA ATA GCA TTA CAG TCC TCA TAA TAG CAT TAC AGT CCT CAT AAT AGC ATT ACA GTC CTC ATA ATA GCA TTA CAG TCC TCA TA	5’Acrydite
C1’ 4C2’ 5’acrydite	AG ACC CTA AGC ATA CAT CGT ATA GAC TAC TGA TAA CTG GAA TAG ACT ACT GAT AAC TGG AAT AGA CTA CTG ATA ACT GGA ATA GAC TAC TGA TAA CTG GA	5’Acrydite

Supplementary Table 6. Fluorophore-tagged DNA for DNA hybridization-based signal amplification (See Supplementary Fig. 10)

Name	Sequence	Modifications
A1 3’alexa488	CG TTG ATG CTT TGT ATT CGG T	3’Alexa488
B2 3’atto565	ACT TAT GAG GAC TGT AAT GCT	3’Atto565
C2 3’atto647N	CAA TCC AGT TAT CAG TAG TCT	3’Atto647N

Supplementary Table 7. Linker DNA for LNA hybridization-based signal amplification (see Supplementary Fig. 10)

Name	Sequence	Modifications
A2’ 4LNA-A1’ 5’Acrydite	GG TGA CAG GCA TCT CAA TCT ATT ACA AAG CAT CAA CGA TTA CAA AGC ATC AAC GAT TAC AAA GCA TCA ACG ATT ACA AAG CAT CAA CG	5’Acrydite
A3’ 4LNA-A1’ 5’Acrydite	GT CCC TGC CTC TAT ATC TCC ATT ACA AAG CAT CAA CGA TTA CAA AGC ATC AAC GAT TAC AAA GCA TCA ACG ATT ACA AAG CAT CAA CG	5’Acrydite
B1’ 4LNA-B2’ 5’Acrydite	TA CGC CCT AAG AAT CCG AAC ATG CAT TAC AGC CCT CAA TGC ATT ACA GCC CTC AAT GCA TTA CAG CCC TCA ATG CAT TAC AGC CCT CA	5’Acrydite

Supplementary Table 8. DNA for triple round expansion

Name	Sequence	Modifications
B1' A2 5'acrydite	TA CGC CCT AAG AAT CCG AAC ATA GAT TGA GAT GCC TGT CAC C	5’Acrydite

Supplementary Table 9. Fluorophore-tagged LNA for LNA hybridization-based signal amplification (see Supplementary Fig. 10) (underlined letters: LNA)

Name	Sequence	Modifications
LNA-A1 3'Atto565	CGTTGATGCTTTGTA	3'Atto565
LNA-B2 3'Atto647N	TGAGGGCTGTAATGC	3'Atto647N

Supplementary Table 10. Immunostaining and DNA hybridization condition

(except **Supplementary Fig. 15**; see '**7. Triple round expansion**' of Methods)

Figure	Imaging method	Primary antibody	Secondary antibody	DNA hybridization after 2nd antibody staining	DNA hybridization after re-embedding	DNA hybridization after 2nd swellable gel synthesis
Main Text Figures						
2a-c	STORM	anti-beta Tubulin (rb)	anti-Rb alexa647			
2d-n	iExM	anti-beta Tubulin (rb)	RbA1'	A1 5'acrydite 3'alexa488	A1' 5'acrydite 3'atto565	
2o,p	STORM/iExM	anti-beta Tubulin (rb)	RbA1' anti-Rb alexa647	A1 5'acrydite 3'alexa488	A1' 5'acrydite 3'atto565	
3a-c	iExM	anti-GluR1 (rb) anti-Basson (ms) anti-Homer1 (gp)	RbA3' MsB1' GpC1'	A3 5'acrydite B1 5'acrydite C1 5'acrydite	A3' 4A1' 5'acrydite B1' 4B2' 5'acrydite C1' 4C2' 5'acrydite	A1 3'alexa488 B2 3'atto565 C2 3'atto647N
3d-f	iExM	anti-GABARAα1/α2 (rb) anti-Gephyrin (ms) anti-Bassoon (gp)	RbA3' MsB1' GpC1'	A3 5'acrydite B1 5'acrydite C1 5'acrydite	A3' 4A1' 5'acrydite B1' 4B2' 5'acrydite C1' 4C2' 5'acrydite	A1 3'alexa488 B2 3'atto565 C2 3'atto647N
3g	iExM	anti-GluR1 (rb) anti-Homer1 (ms)	RbA2' MsB1'	A2 5'acrydite B1 5'acrydite	A2' 4LNA-A1' 5'acrydite B1' 4LNA-B2' 5'acrydite	LNA-A1 3'atto565 LNA-B2 3'atto647N
3i-s	iExM	anti-Homer1 (rb) anti-Bassoon (ms)	RbA2' MsB1'	A2 5'acrydite B1 5'acrydite	A2' 4LNA-A1' 5'acrydite B1' 4LNA-B2' 5'acrydite	LNA-A1 3'atto565 LNA-B2 3'atto647N
3t	iExM	anti-Homer1 (rb) anti-mCherry (rt)	RbA2' RtC1'	A2 5'acrydite C1 5'acrydite	A2' 4LNA-A1' 5'acrydite C1' 5'acrydite 3'alexa488	LNA-A1 3'atto565
3u	iExM	anti-Homer1 (rb) anti-GFP (chk)+	RbA2' ChkC1'	A2 5'acrydite C1 5'acrydite	A2' 4LNA-A1' 5'acrydite C1' 5'acrydite 3'alexa488	LNA-A1 3'atto565
4a		anti-GFP (chk)+ anti-mTFP (rt) anti-TagRFP (gp)*	anti-Chk alexa488 anti-Rt alexa546 anti-Gp CF633			
4b	ProExM	anti-GFP (chk)+ anti-mTFP (rt) anti-TagRFP (gp)*	anti-Chk alexa488 anti-Rt alexa546 anti-Gp CF633			

4c	iExM	anti-mCherry (rb) anti-TagRFP (gp)* anti-GFP (chk)$^{+}$ anti-mTFP (rt)	RbA2' GpB1' ChkC1' RtC1'	A2 5'acrydite B1 5'acrydite C1 5'acrydite	A2' 4LNA-A1' 5'acrydite B1' 4LNA-B2' 5'acrydite C1' 5'acrydite 3'alexa388	LNA-A1 3'atto565 LNA-B2 3'atto647N
4d	iExM	anti-mCherry (rb) anti-TagRFP (gp)* anti-GFP (chk)$^{+}$ anti-mTFP (rt)	RbA2' GpB1' ChkC1' RtC1'	A2 5'acrydite B1 5'acrydite C1 5'acrydite	A2' 4LNA-A1' 5'acrydite B1' 4LNA-B2' 5'acrydite C1' 5'acrydite 3'alexa388	LNA-A1 3'atto565 LNA-B2 3'atto647N
Supplementary Information Figures						
1	hp-iExM	anti-beta Tubulin	RbA1'	A1 5'acrydite 3'alexa488	A1' 5'acrydite 3'atto565	
2b	iExM	anti-beta Tubulin (rb)	RbA1'	A1 5'acrydite 3'alexa488	A1' 5'acrydite 3'atto565	
7	iExM	anti-beta Tubulin (rb)	RbA1'	A1 5'acrydite 3'alexa488	A1' 5'acrydite 3'atto565	
9	iExM	anti-Homer1 (rb) anti-Bassoon (ms)	MsA2' RbB1'	A2 5'acrydite B1 5'acrydite	A2' 4LNA-A1' 5'acrydite B1' 4LNA-B2' 5'acrydite	LNA-A1 3'atto565 LNA-B2 3'atto647N
10	iExM	anti-GABARAα1/α2 (rb) anti-Bassoon (ms)	RbA2' MsB1'	A2 5'acrydite B1 5'acrydite	A2' 4LNA-A1' 5'acrydite B1' 4LNA-B2' 5'acrydite	LNA-A1 3'atto565 LNA-B2 3'atto647N
11	same with **Fig. 4c**					
12,13	iExM	anti-mCherry (rb) anti-TagRFP (gp)* anti-mTFP (rt)	RbA2' GpB1' RtC1'	A2 5'acrydite B1 5'acrydite C1 5'acrydite	A2' 4LNA-A1' 5'acrydite B1' 4LNA-B2' 5'acrydite C1' 5'acrydite 3'alexa388	LNA-A1 3'atto565 LNA-B2 3'atto647N
14	hp-iExM	anti-TagRFP (gp)* anti-mTFP (rt) anti-GFP (chk)$^{+}$	GpA2' RtB1' ChkC1'	A2 5'acrydite B1 5'acrydite C1 5'acrydite	A2' 4LNA-A1' 5'acrydite B1' 4LNA-B2' 5'acrydite C1' 5'acrydite 3'alexa388	LNA-A1 3'atto565 LNA-B2 3'atto647N

*anti-TagRFP antibody binds also to TagBFP.
$^{+}$anti-GFP antibody binds also to EYFP.

Supplementary Table 11. Primary and secondary antibody list

Primary/ Secondary	Target	Host*	Vendor**	Product number	Dilution
Primary	Beta tubulin	Rb	Abcam	ab6046	1:100
Primary	Homer1	Rb	SYSY	160003	1:200
Primary	Homer1	Gp	SYSY	160004	1:200
Primary	Homer1	Ms	SYSY	160011	1:200
Primary	Bassoon	Ms	Enzo	ADI-VAM-PS003-F	1:200
Primary	Bassoon	Gp	SYSY	141004	1:200
Primary	Gephyrin	Ms	SYSY	147011	1:200
Primary	$GABA_AR\alpha1$	Rb	SYSY	224203	1:200
Primary	$GABA_AR\alpha2$	Rb	SYSY	224103	1:200
Primary	GluR1	Rb	Abcam	ab31232	1:100
Primary	TagRFP	Gp	Kerafast/Cai lab	EMU107	1:200
Primary	mCherry	Rb	Abcam	ab167453	1:200
Primary	mCherry	Rt	ThermoFisher	M11217	1:200
Primary	mTFP	Rt	Kerafast/Cai lab	EMU103	1:200
Primary	GFP	Chk	Kerafast/Cai lab	EMU101	1:400
Secondary	Chicken	Gt	ThermoFisher	A-11039 (alexa 488 conjugated)	10 µg/uL
Secondary	Rat	Gt	ThermoFisher	A-11081 (alexa 546 conjugated)	10 µg/uL
Secondary	Guinea Pig	Gt	Biotium	Biotium (CF633 conjugated)	10 µg/uL
Secondary	Rabbit	Dk	JIR	711-005-152	10 µg/uL for cultured cells and cultured neurons and 20 µg/µL for brain slices
Secondary	Chicken	Dk	JIR	703-005-155	
Secondary	Rat	Dk	JIR	712-005-153	
Secondary	Guinea Pig	Dk	JIR	706-005-148	
Secondary	Mouse	Dk	JIR	715-005-151	
Secondary	Rabbit	Dk	ThermoFisher	A-31573 (alexa 647 conjugated)	1:100

*Host - Rb: rabbit, Ms: mouse, Gp: Guinea pig, Rt: Rat, Chk: Chicken, Gt: Goat, Dk: Donkey
**Vender – SYSY: Synaptic Systems, JIR: Jackson ImmunoRearch

Supplementary Table 12. DNA-conjugated secondary antibodies

Name	Host of 2nd antibody	Conjugated DNA
RbA1'	Rabbit	A1' 5'amine
RbA2'	Rabbit	A2' 5'amine
RbA3'	Rabbit	A3' 5'amine
RbB1'	Rabbit	B1' 5'amine
MsA2'	Mouse	A2' 5'amine
MsB1'	Mouse	B1' 5'amine
GpA2'	Guinea pig	A2' 5'amine
GpB1'	Guinea pig	B1' 5'amine
GpC1'	Guinea pig	C1' 5'amine
RtB1'	Rat	B1' 5'amine
RtC1'	Rat	C1' 5'amine
ChkC1'	Chicken	C1' 5'amine

Supplementary Table 13. Gel solution of hp-iExM and iExM

	Pre-gel Incubation solution	1st gel solution	Re-embedding solution	hp-iExM 2nd gel solution	iExM 2nd gel solution
Sodium acrylate	8.625% (w/w)	8.625% (w/w)	0	0	8.625% (w/w)
Acrylamide	2.5% (w/w)	2.5% (w/w)	10% (w/w)	10% (w/w)	2.5% (w/w)
Crosslinker	DHEBA 0.2% (w/w)	DHEBA 0.2% (w/w)	DHEBA 0.2% (w/w)	BIS 0.15% (w/w)	BIS 0.15% (w/w)
APS	0	0.2% (w/w)	0.05% (w/w)	0.05% (w/w)	0.05% (w/w)
TEMED	0	0.2% (v/w)	0.05% (v/w)	0.05% (v/w)	0.05% (v/w)
NaCl	1.865M	1.865M	0	0	2M
PBS	1x	1x	0	0.15x	1x
H-tempo	0	0.005%	0	0	0
Incubation/gelation temperature	4 ℃	37 ℃	37 ℃	Room temperature	37 ℃
Incubation/gelation duration	Overnight (12 hours)	3 hours	1.5 hours	1.5 hours	1.5 hours

Supplementary Table 14. Gel solution for triple round expansion

	Pre-gel Incubation solution	1st gel solution	1st Re-embedding solution	2nd gel solution	2nd re-embedding solution	3rd gel solution
Sodium acrylate	8.625% (w/w)	8.625% (w/w)	0	8.625% (w/w)	0	8.625% (w/w)
Acrylamide	2.5% (w/w)	2.5% (w/w)	10% (w/w)	2.5% (w/w)	10% (w/w)	2.5% (w/w)
Crosslinker	BAC 0.2% (w/w)	BAC 0.2% (w/w)	BAC 0.2% (w/w)	DHEBA 0.2% (w/w)	DHEBA 0.2% (w/w)	BIS 0.15% (w/w)
APS	0	0.2% (w/w)	0.05% (w/w)	0.05% (w/w)	0.05% (w/w)	0.05% (w/w/)
TEMED	0	0.2% (v/w)	0.05% (v/w)	0.05% (v/w)	0.05% (v/w)	0.05% (v/w)
NaCl	1.11M	0.89M	0	2M	0	2M
PBS	1x	1x	0	1x	0	1x
H-tempo	0	0.005%	0	0	0	0
Incubation/Gelation temperature	4 ℃	37 ℃	37 ℃	37 ℃	37 ℃	37 ℃
Incubation/Gelation duration	Overnight (12 hours)	3 hours	1.5 hours	1.5 hours	1.5 hours	1.5 hours

Supplementary Note 1. Expansion factor: We found that iExM would typically result in expansion ratios of ~4.5x (with 0.005% H-TEMPO inhibitor; see Methods for details) to ~5.5x (with 0.01% H-TEMPO) in the first round, and ~4x in the second round, for a total increase of ~16x-22x. hp-iExM resulted in ~4.5x (with 0.005% H-TEMPO) to ~5.5x (with 0.01% H-TEMPO) expansion ratios in the first round of expansion, followed by ~3.5x in the second round, for a total increase of ~14-19x.

Supplementary Note 2. Details of the iExM simulator: We developed a computer simulation of how microtubules would look when they were labeled with a primary antibody and a DNA-conjugated secondary antibody, and then expanded 20-fold via iExM (**Supplementary Fig. 2**). We first calculated the point spread function (PSF) of the microscope system we used (a spinning disk confocal microscope with a pinhole size of 50 μm equipped with a 40x NA1.15 objective lens), as shown in **Supplementary Fig. 2a**. We positioned points in a cylinder between two shells, with inner and outer radius R_i and R_o respectively. The points in the cylinder simulate the distribution of the 5' acrydites of the DNA anchored to the first polymer of iExM. This DNA is anchored to the hydrogel through its 5' acrydite, and then a complementary DNA with a fluorophore is finally hybridized to this gel-anchored DNA, for incorporation into the final gel. These points were randomly positioned inside the cylinder to simulate the stochastic nature of the antibody binding to its target protein. We then convolved the PSF with the points in the cylinder to construct a final image of a microtubule. See section 'MATLAB simulation of iExM images' of the Methods for details of calculating R_i and R_o. A simulated cylinder with an inner radius of the average R_i and outer radius of the average R_o (green dots, **Supplementary Fig. 2c, 2d**) was generated and super-imposed onto the experimental results (red dots). When we analyzed 129 microtubule segments (from one culture), the average R_i was 26.7 ± 5.6 nm (mean ± standard deviation) and the average R_o was 33.5 ± 2.1 nm, as shown in **Supplementary Fig. 2e**. The average thickness of the 5' acrydite layer was 6.8 ± 3.6 nm, as shown in **Supplementary Fig. 2f**.

Supplementary Note 3. Positional error of proteins labeled with DNA-conjugated secondary antibodies: We can (**Supplementary Fig. 4**) calculate the positional error when a DNA-conjugated secondary antibody is used instead of a regular secondary antibody to label a protein complex on two sides, as shown in **Supplementary Fig. 4c** and **d**. The peak-to-peak distance would be 60.2 nm (**Supplementary Fig. 4c** and green trace in **d**); taking the peak to be at the center of the DNA layer, estimated using the calculations of **Supplementary Fig. 2e,** or 2 × (26.7+33.5)/2), which is 9.2 nm larger than the peak-to-peak distance (51 nm) measured by immunostaining with regular secondary antibodies. So, the DNA-conjugated antibody adds 4.6 nm (9.2/2=4.6 nm per epitope) of positional error to a protein complex vs. when labeled with regular secondary antibodies if the antibodies bind to the target protein asymmetrically, as in **Supplementary Figure Fig. 4a** and **d**.

Supplementary Note 4. New probe designs with a smaller probe size: In our experiments, a secondary antibody conjugated with a 7-nm long strand of DNA was used (**Supplementary Fig. 5a**). As this DNA-antibody complex is larger than a regular secondary antibody (**Supplementary Fig. 5b**), the use of a DNA-conjugated antibody adds another 4.6 nm positional error to a typical measurement, as discussed in **Supplementary Fig. 4**. We schematized (**Supplementary Fig. 5**) three options to make the DNA-conjugated secondary antibody smaller. First, a shorter strand of DNA could be used (**Supplementary Fig. 5c**). To prevent the melting of shorter double strand DNA oligos, a buffer with a higher salt concentration could be used. Second, the position of an acrydite moiety could be changed from the far end of the DNA to the proximal end (**Supplementary Fig. 5d**). Thirdly, a single stranded DNA oligo with an acrydite moiety could be conjugated to the secondary antibody directly. As the persistence length of single stranded DNA is much shorter than double stranded DNA[2], the distance from the surface of the secondary antibody to the acrydite would be shorter than the current design (**Supplementary Fig. 5e**). All three options presented here use only commercially available reagents, and would not require additional modification to the current iterative expansion microscopy protocol. To minimize such positional errors further, one could use nanobodies (camelid nanobodies, or F(ab) fragments of secondary antibodies) and/or direct conjugation of DNA to a primary antibody. By combining these two options (antibody fragments or direct conjugation of DNA to primary antibodies) with the three options for DNA-antibody conjugation strategies presented above, iExM with much smaller structural errors might be possible.

Supplementary Note 5. Resolution measurement: We estimated the point spread function (PSF) of iExM by measuring the full width at half maximum (FWHM) of single microtubule sidewalls, deriving a value of 25.8 ± 7.7 nm (**Supplementary Fig. 6a**). This number was comparable to the FWHM of single microtubule sidewalls measured by other super-resolution microscopies (16-25 nm for 4Pi single-molecule switching nanoscopy (4PiSMSN) imaging of microtubules with a regular secondary antibody[4] and 21-27 nm for buffer-enhanced STORM imaging of microtubules with an antibody fragment[5]). We estimated the point spread function (PSF) of iExM independent of the labels (primary antibody and DNA-conjugated secondary antibody) by deconvolving images of microtubule sidewalls by idealized microtubules (generated according to the model of **Supplementary Fig. 3**) bearing primary antibodies and DNA-conjugated secondaries (but not modeling the blur due to optical diffraction). This yielded a value of 22.3 nm ± 5.3 nm (**Supplementary Fig. 6b**) for the contribution to the PSF due to the gelation, expansion, and optical imaging processes.

In iterative expansion microscopy, specimens are expanded 20-fold, but the microscope resolution is not improved by exactly 20-fold because the gelation and expansion process may introduce error. We can estimate the magnitude of this error by simulating (using the iExM simulator described in **Supplementary Fig. 2**, which includes optical blur due to diffraction) the FWHM of single microtubule sidewalls; we obtain 19.4 ± 1.4 nm for this FWHM, which models the case where microtubules are labeled with a primary antibody and DNA-conjugated secondary antibody and then expanded 20-fold without gel-related error (**Supplementary Fig. 6a**). Thus, the experimental PSF (25.8 nm, **Supplementary Fig. 6a**) is ~6 nm larger than the simulated value. Why is this? Before expansion, polymer chains form a dense polymer network with a mesh size of a few nanometers (small angle x-ray scattering measurements of similar gels suggest a mesh size of

1-2 nm, ref. [6]). During gelation and expansion, the 5' acrydite moieties would be anchored to nearby polymer chains, which would "coarse grain" the set of possible acrydite anchoring points (since acrydites could not be anchored at a point in space unless a gel chain was present). During expansion itself, gels may not move perfectly evenly, as well. Such broadening would not greatly alter the mean peak-to-peak distance between target proteins arranged in a stereotyped complex, because the errors of 5'acrydite tags would be averaged out in the final images (**Supplementary Fig. 6c**). However, these gel effects would broaden the PSF of iExM by randomly moving 5'acrydite tags from their initial positions.

To gauge the impact of such errors on our original simulation (**Supplementary Fig. 2**), we incorporated errors of this scale into the simulation of **Supplementary Fig. 2**, by randomly moving 5' acrydites in the cylinder by 5-10 nm relative to their initial positions and performing the simulation of **Supplementary Fig. 2a** again. As shown in **Supplementary Fig. 6c**, an additional 5-10 nm positional error did not greatly alter the overall microtubule profile, shifting the peak-to-peak distance between the sidewalls by a few nanometers.

Supplementary Note 6. Expansion uniformity: Expansion uniformity across length scales of tens to hundreds of nanometers can be estimated by analyzing how the microtubule diameter varies along the long axis of the microtubule. Before expansion, this variation would be a result of two factors: variation in the actual microtubule diameter along the microtubule long axis before expansion, and variation in the thickness of the primary and secondary antibody layers along the microtubule long axis. If expansion was perfectly uniform, then the variation in the microtubule diameter along the microtubule long axis after expansion would be the pre-expansion variation, scaled up by the expansion factor. However, if the expansion was not uniform, then this variation might be larger than expected from a simple scaling.

By comparing the standard deviation of the microtubule diameter along the microtubule long axis as measured in STORM imaging vs. iExM imaging, we can calculate the nonuniformity of expansion across length scales of tens to hundreds of nanometers. The standard deviation of the peak-to-peak sidewall distances measured by STORM, over distances of 400 nm along the microtubule axis, was 4.7 nm (n = 110 from two cultures). This standard deviation was 10.3 nm (in scaled-down-to-pre-expansion units) when measured by iExM imaging of cultured cells (n = 307 from one cultures), and 10.5 nm when measured by hp-iExM imaging of cultured cells. The difference of variances between iExM and STORM ($(10.3^2 - 4.7^2)^{1/2}$ = 9.2 nm deviation) could be attributed to the extra DNA layer used in ExM but not STORM, as well as any non-uniformity of expansion. We calculated this standard deviation of post-expansion sidewall distances of microtubules in tissues, obtaining 13.5 nm for the brain (n = 96 from one samples), 11.5 nm for the lung (n = 55 from one samples), and 13.6 nm for the liver (n = 95 from one samples). The difference of variances (e.g., (13.6^2 nm – $4.7^2)^{1/2}$ = 12.8 nm deviation for the liver) again would be attributed to the iExM-specific properties – the DNA layer and non-uniform expansion. It is nontrivial to precisely separate the effects of DNA layer and non-uniform expansion from these measurements and calculations.

Supplementary Note 7. Discussion on the size of the linker DNA: For DNA amplification (**Supplementary Fig. 8**), the final expansion occurred in 0.2x PBS to maintain DNA hybridization, although this resulted in less expansion than distilled (DI) water. For LNA, the second gel was expanded in DI water, enabling full expansion, and made possible by the strong hybridization between LNA and DNA which survives immersion in DI water[3]. The use of a long linker DNA, and then hybridizing multiple DNA or LNA strands to the linker DNA, would not add a large error to the location of biological targets, as the DNA and LNA strands are hybridized to the linker DNA after the 2nd expansion, so that the effective positional error, calculated by dividing the positional error by the expansion factor, is negligible (for example, the length of a fully stretched 100-bp linker DNA is expected to be around 33 nm, but the effective positional error caused by this linker DNA would be only 33 / 20 ~ 1.7 nm).

Supplementary Note 8. Triple round expansion: We designed a triple expansion protocol (**Supplementary Fig. 15**) where the first swellable gel uses the disulfide-containing crosslinker N,N'-cystaminebisacrylamide (which can be cleaved with tris(2-carboxyethyl)phosphine (TCEP)), the second swellable gel uses the diol-containing crosslinker DHEBA (which can be cleaved with NaOH), and the third swellable gel uses the standard crosslinker BIS (which is resistant to both TCEP and NaOH). We expect that a challenge to using iterative expansion microscopy with a larger expansion factor (>50 fold) would be validating the nanoscale expansion uniformity. To validate 20-fold expansion, we used microtubules as molecular rulers, but even smaller structures would be required to validate the resolution of 50-fold expansion. One possible option would be DNA origami, as a wide range of DNA origami structures are available[7] and large probes (e.g., antibodies) are not required to anchor them to the gel.

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