Electronic Supporting Information

FRACTAL: Signal amplification of immunofluorescence *via* cyclic staining of target molecules

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Experimental Methods

All the materials are listed in Supplementary Table 2. Detailed staining protocols are summarized in Supplementary Table 3.

Cell culture. BS-C-1 and HeLa cells were obtained from the Korean Cell Line Bank and seeded into Nunc Lab-Tek II chambers. HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% penicillin-streptomycin and 10% Fetal Bovine Serum (FBS). BS-C-1 cells were cultured in Modified Eagle's Medium (MEM) supplemented with 1% penicillin-streptomycin, 1% sodium pyruvate, and 10% FBS. All cells were maintained in an incubator at 37 ℃ in 5% CO_2.

Cell fixation. For staining of tubulin, BS-C-1 cells were first extracted for 30 s with cytoskeleton extraction buffer (0.1 M 1,4-piperazinediethanesulfonic acid (PIPES), 1 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 200 mM sodium hydroxide (NaOH), 1 mM magnesium chloride ($MgCl_2$), 0.2% Triton X-100, pH 7) and then fixed with tubulin fixation solution (3% paraformaldehyde (PFA), 0.1% glutaraldehyde (GA), 1× phosphate-buffered saline (PBS)) for 10 min, followed by a reduction with 0.1% sodium borohydride in 1× PBS for 6 min, and then rinsed with 0.1 M glycine in 1× PBS three times. For staining of proteins other than tubulin, cells were fixed with 4% PFA in 1× PBS for 10 min and rinsed with 1× PBS three times, 5 min each time.

Mouse brain perfusion and dissection. All of the following animal experiments were approved by the Korea Advanced Institute of Science and Technology Institutional Animal Care and Use Committee (KAIST-IACUC). Mice used in this study were maintained in a specific pathogen-free facility of KAIST Laboratory Animal Resource Center. Mice (C57BL/6J, ages of 8–14 weeks) were perfused with 4% PFA in 1× PBS. The brains were harvested and further fixed with 4% PFA in 1× PBS for 2 h at 4 ℃. The brains were dissected to a thickness of 150 μm and stored in storage buffer (0.01% sodium azide, 0.1 M glycine, 1× PBS) at 4 ℃ until needed.

Conjugating antibodies to fluorophores. First, 90 μL of an antibody solution with a concentration of 1 mg/ml was mixed with sodium bicarbonate buffer (1 M, 10 μL, pH 8.3). Second, NHS-ester fluorophore in dimethyl sulfoxide (DMSO) was added to the mixture, with a molar ratio of NHS-ester fluorophore to an antibody 9:1. The solution was incubated for 1 h at room temperature. The fluorophore-conjugated antibodies were separated from the unreacted fluorophores and antibodies through NAP-5 gel filtration columns. Before filtration, the columns were equilibrated with 1× PBS. Incubated fluorophore-conjugated antibodies solutions were added to the columns with 400 μL of 1× PBS. As two fluorescent bands were separated, 500 μL of 1× PBS was loaded into the columns. The eluates were concentrated using centrifugal filter with 100,000 molecular weight cut-off (MWCO), as instructed by a manufacturer.

FRACTAL immunostaining of cells and mouse brain slices. All of the following procedures were performed at RT. For all the following staining processes, a primary antibody was diluted to 2.5 μg/mL in MAXbind™ Staining Medium, and secondary antibodies were diluted to 5.2 μg/mL in MAXbind™ Staining Medium. All washing steps were performed with MAXwash™ Washing Medium. Fixed cells were first blocked in MAXblock™ Blocking Medium for 2 h and washed three times. For the primary antibody staining, cells were stained with rabbit anti-lamin B1 primary antibody for 1 h and then washed three times. For the first secondary antibody staining, cells were stained with CF633-conjugated donkey anti-rabbit secondary antibody for 1 h and washed three times. For the second secondary antibody staining, cells were stained with CF633-conjugated rabbit anti-donkey secondary antibody for 1 h and

washed three times. These two secondary antibody staining steps were repeated to amplify the signals. For the staining of mouse brain slices, the brain slices were incubated with a rabbit primary antibody against GFAP diluted to 0.125 μg/mL in MAXbind™ Staining Medium. The first and second secondary staining steps were identical to the cultured cell protocol except for the incubation time (blocking: 2 h, staining: 1 h, washing: three times, 30 min each time).

FRACTAL immunostaining of tubulin in cultured cells for STORM imaging. Cells were extracted and fixed using cytoskeleton extraction and tubulin fixation protocol in the previous method session. With the same staining procedure described in the previous part, a primary antibody was diluted to 2.5 μg/mL in MAXbind™ Staining Medium, and secondary antibodies were diluted to 5.2 μg/mL in MAXbind™ Staining Medium. All washing steps were performed with MAXwash™ Washing Medium. Fixed Cells were blocked in MAXblock™ Blocking Medium for 2 h and then washed three times. For the primary antibody staining, cells were stained with rabbit anti-α-tubulin primary antibody for 1 h and washed three times. For the first secondary antibody staining, cells were stained with donkey anti-rabbit secondary antibody for 1 h. For the second secondary antibody staining, cells were stained with a rabbit anti-donkey secondary antibody for 1 h and then washed three times. These two secondary antibody staining steps were repeated to amplify the signals. At the final staining step, cells were stained with Alexa Fluor 647-conjugated goat anti-rabbit secondary antibody diluted to 8 μg /mL, instead of donkey anti-rabbit secondary antibody, for 1 h and then washed three times.

Multicolor FRACTAL immunostaining of cells. With the same staining procedure described in the previous part, a primary antibody was diluted to 2.5 μg/mL in MAXbind™ Staining Medium, and secondary antibodies were diluted to 5.2 μg/mL in MAXbind™ Staining Medium. All washing steps were performed with MAXwash™ Washing Medium. The fixed cells were blocked in MAXblock™ Blocking Medium for 2 h and then washed three times. For the multicolor imaging, lamin B1, vimentin, and tubulin of cultured cells were stained with primary antibody and fluorophore-conjugated secondary antibody. As primary antibody staining, cells were stained with rabbit anti-lamin B1, chicken anti-vimentin, and rat anti-tubulin primary antibodies for 1 h and washed three times. For the secondary antibody staining, signal amplification of lamin B1 was processed first, and then the secondary antibody staining of vimentin, and tubulin was processed. For the first secondary antibody staining, cells were stained with CF 633-conjugated donkey anti-rabbit secondary antibody for 1 h. For the second secondary antibody staining, cells were stained with CF 633-conjugated rabbit anti-donkey secondary antibody for 1 h and then washed three times. These two secondary antibody staining steps were repeated four times. Then, vimentin and tubulin were simultaneously stained with CF 568-conjugated goat anti-chicken and CF 488-conjugated mouse anti-rat secondary antibodies for 1 h and then washed three times.

Multicolor FRACTAL immunostaining of FFPE samples. All of the following human sample experiments were approved by the Korea Advanced Institute of Science and Technology Institutional Review Board (KAIST IRB). The formalin-fixed paraffin-embedded (FFPE) samples were obtained from Novus Biologicals (NBP2-30189). The samples were deparaffinized in xylene twice, and then hydrated in 100% ethanol twice, 95% ethanol once, 80% ethanol once, and deionized water (DI) once. Each of these treatments was performed for 3 min. The samples were then placed in a retrieval solution (20 mM sodium citrate solution, pH 8.0), which was preheated to approximately 100 ℃, and then incubated in a 60 ℃ chamber for 30 min. For the staining of the FFPE samples, the blocking, staining, and washing steps were identical to the cultured cell protocol, except for the incubation time (blocking: 2 h, staining: 1 h, washing: three times, 10 min each time).

Protein-retention expansion microscopy; **gelation, digestion, and expansion.** The stained cells were treated with 0.1 mg/mL of acryloyl-X, SE (6-((acryloyl)amino) hexanoic acid, succinimidyl ester, in 1× PBS) for 1 h at room temperature, and washed three times with 1× PBS. The cells were then incubated with a gelation solution (2.5% (w/w) acrylamide, 7.5% (w/w) sodium acrylate, 0.15% (w/w) *N,N′*-methylenebisacrylamide, 2 M sodium chloride (NaCl), 0.2% (w/w) ammonium persulfate (APS), 0.2% (w/w) tetramethylethylenediamine (TEMED), and 0.01% (w/w) 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (H-TEMPO), 1× PBS) at 4 ℃ for 30 min and 37 ℃ for 1 h. After gelation, the cells were incubated with a digestion buffer (1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris (pH 8), 0.5 % Triton X-100, 1 M NaCl, 8 units/ml proteinase K) for 1 h at 37 ℃. After digestion, the gels were placed in DI water. DI water was exchanged every 15 min 3–4 times with gentle shaking. For the protein-retention expansion microscopy of mouse brain slices, AcX treatment, gelation, digestion, and washing steps were identical to the cultured cell protocol except for the incubation time (AcX treatment: overnight, incubation with a gelation solution at 4 ℃: 30 min, gelation: 1.5 h, digestion: overnight, washing: 30 min for each).

Ultrastructures using expansion microscopy (U-ExM); gelation, digestion, expansion, and post-expansion staining. The brain slices were fixed with fixation solution (0.7% (w/w) formaldehyde, 1% (w/w) acrylamide, 1× PBS) at 37 ℃ for 5 h. The brain slices were then incubated with a gelation solution (10% (w/w) acrylamide, 19% (w/w) sodium acrylate, 0.1% (w/w) *N,N′*-methylenebisacrylamide, 2 M sodium chloride (NaCl), 0.5% (w/w) ammonium persulfate (APS), 0.5% (w/w) tetramethylethylenediamine (TEMED), and 0.025% (w/w) 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (H-TEMPO), 1× PBS) at 4 ℃ for 30 min and 37 ℃ for 1 h. After gelation, the gels were incubated with a denaturation buffer (200 mM sodium dodecyl sulfate (SDS), 200 mM NaCl, and 50 mM Tris in DI at pH 9) for 15 min at RT. The gels were moved to a fresh denaturation buffer and incubated for 1 h 30 min at 95 ℃. After denaturation, the gels were placed in DI water overnight. Before blocking, the gels were placed in 1× PBS twice for 15 min with gentle shaking. The gels were first blocked in MAXblock™ Blocking Medium for 3 h and washed three times. For the primary antibody staining, cells were stained with goat anti-GFAP primary antibody for 1 h and then washed three times. For the first secondary antibody staining, cells were stained with CF 568-conjugated chicken anti-goat secondary antibody for 3 h and washed three times (10 min for each). For the second secondary antibody staining, cells were stained with a CF 568-conjugated goat anti-chicken secondary antibody for 3 h and washed three times. These two secondary antibody staining steps were repeated to amplify the signals. After staining, the gels were placed in DI water. DI water was exchanged every 15 min 3–4 times with gentle shaking before imaging. For all the staining processes, a primary antibody was diluted to 0.125 μg/mL in MAXbind™ Staining Medium, and secondary antibodies were diluted to 5.2 μg/mL in MAXbind™ Staining Medium. All washing steps were performed with MAXwash™ Washing Medium.

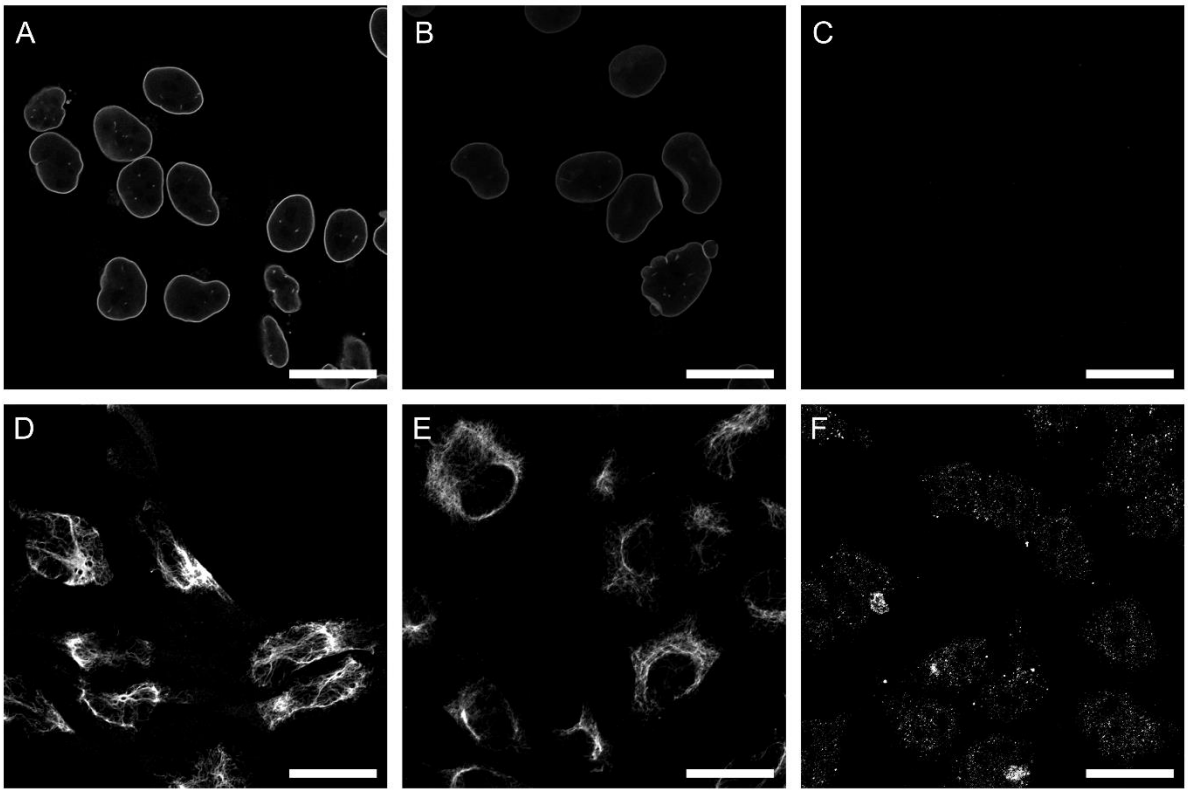
Imaging. Images shown in Figure 1(C–D) and 3(B–K) were acquired by a Nikon C2 plus equipped with a 40×, 1.15 NA, water-immersion objective. Images shown in Figure 4(B–E) were acquired by an Andor spinning disk, equipped with the same objective.

STORM imaging. All STORM experiments were performed on an inverted microscope (Ti2-U; Nikon) equipped with an objective lens 100× (CFI SR HP Apo TIRF; Nikon), 1.49 NA oil immersion. The samples were imaged in an imaging buffer supplemented with 100 mM mercaptoethylamine (MEA), 5% glucose (wt/vol), and oxygen scavenging enzymes (0.5 mg/mL glucose oxidase, and 38 μg/mL catalase) in PBS at pH 8.5. Alexa Fluor 647 dyes were excited by 100 mW 647 nm laser (OBIS;

Coherent) and activated by 0.1–1 mW 405 nm laser (OBIS; Coherent). Output fluorescence was filtered with a bandpass emission filter (LF408/488/561/635-B; Semrock) and imaged onto an electron-multiplying charge-coupled device (EMCCD) camera (iXon Ultra 888; Andor) at a frame rate of 60 Hz. The focus was stabilized during data acquisition using the CRISP Autofocus system (ASI). For three-dimensional (3D) STORM imaging, a cylindrical lens with a focal length of 500 mm (LJ1144RM-A; Thorlabs) was inserted into the detection path to create astigmatism into the point-spread function for determining the x, y and z positions of molecules.

Determination of the degree of signal amplification. To determine the degree of signal amplification in Supplementary Figure 2–4, images were acquired at the same location of the specimens after different staining steps. The images were then registered by using ImageJ. Line-intensity profiles perpendicular to the nuclear membrane were measured from the images, and the degree of signal amplification at different staining steps was calculated.

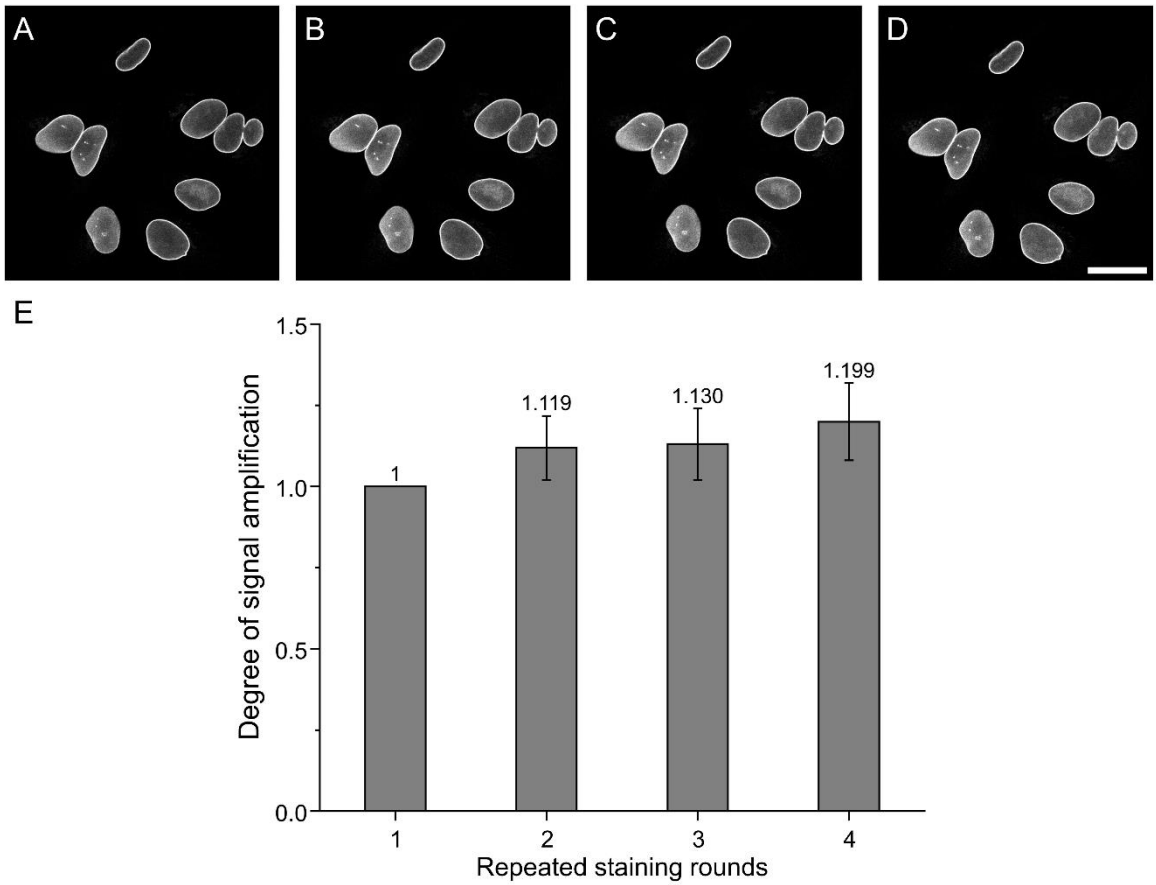
Supplementary Figures



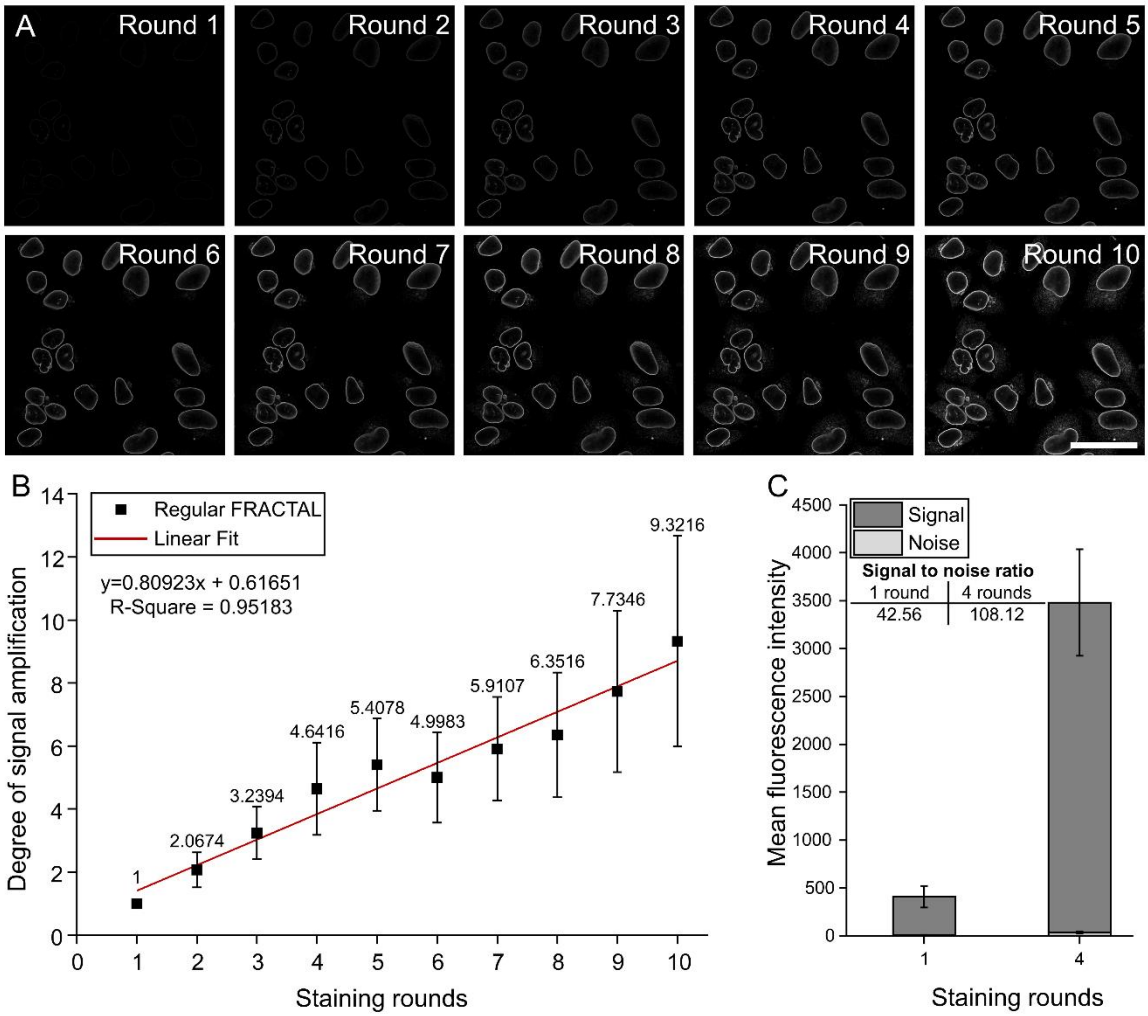
Primary antibody: rabbit anti-lamin B1 (Subfigure A—C, above)		
Buffer \ Staining Step	After first secondary antibody (donkey anti-rabbit) staining	After second secondary antibody (rabbit anti-donkey) staining
MAXblock™ for blocking MAXbind™ for staining MAXwash™ for washing	Stained (Image not shown)	Signal amplified (Subfigure A, above)
5% Normal donkey serum + 0.2% Triton + 1× PBS for blocking, staining, and washing	Stained (Image not shown)	Signal not amplified (Subfigure B, above)
5% Normal rabbit serum + 0.2% Triton + 1× PBS for blocking, staining, and washing	No signal (Image not shown)	No signal (Subfigure C, above)
Primary antibody: chicken anti-vimentin (Subfigure D—F, above)		
Buffer \ Staining Step	After first secondary antibody (goat anti-chicken) staining	After second secondary antibody (chicken anti-goat) staining
MAXblock™ for blocking MAXbind™ for staining MAXwash™ for washing	Stained (Image not shown)	Signal amplified (Subfigure D, above)
5% Normal goat serum + 0.2% Triton + 1× PBS for blocking, staining, and washing	Stained (Image not shown)	Signal not amplified (Subfigure E, above)
5% Normal chicken serum + 0.2% Triton + 1× PBS for blocking, staining, and washing	No signal (Image not shown)	Only non-specific binding (Subfigure F, above)

Supplementary Figure 1. FRACTAL with different staining buffers. HeLa cells were stained with a primary antibody, first secondary antibody, and second secondary antibody in three different buffers. Confocal microscopy images were acquired after the second secondary antibody staining. (A–C) Primary antibody: rabbit antibody against lamin B1, first secondary antibody: CF 633-conjugated

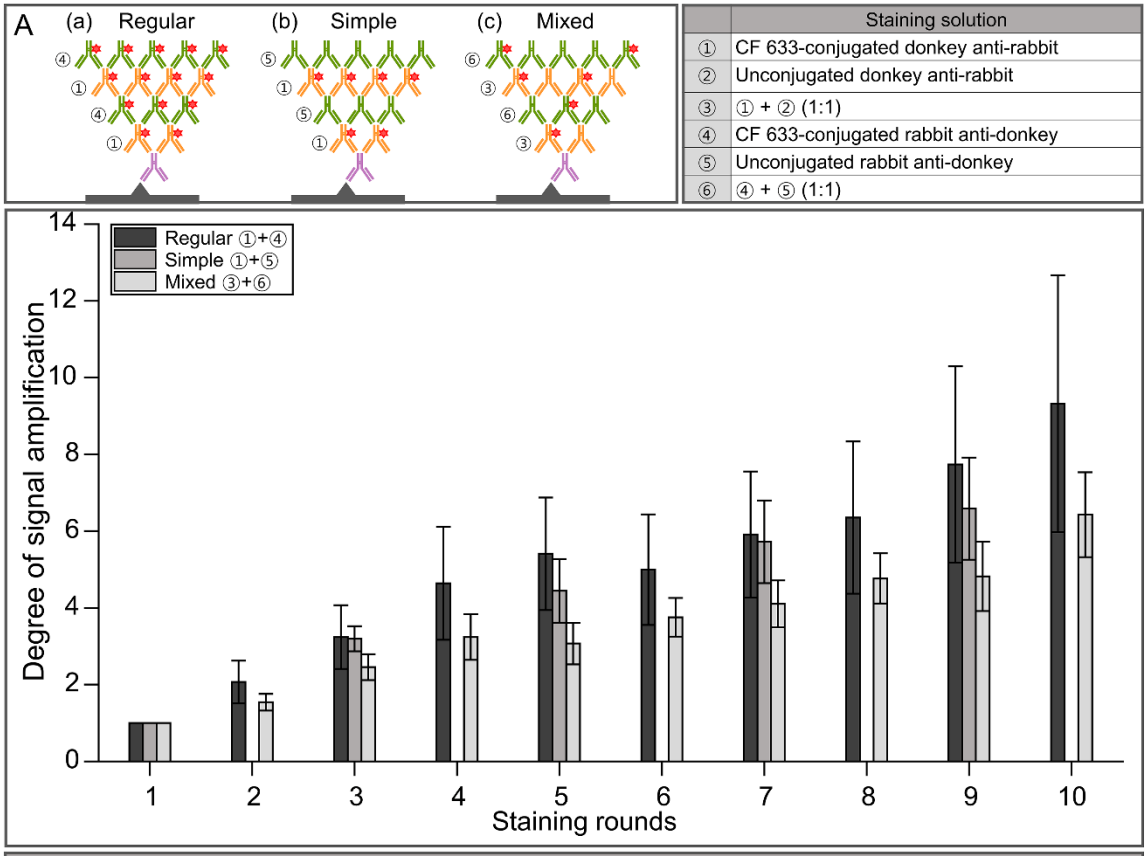
donkey anti-rabbit, second secondary antibody: CF 633-conjugated rabbit anti-donkey. (D–F) Different HeLa cells, stained with a different set of antibodies. Primary antibody: chicken antibody against vimentin, first secondary antibody: CF 568-conjugated goat anti-chicken, second secondary antibody: CF 568-conjugated chicken anti-goat. (A,D) The cells were blocked in MAXblock™ Blocking Medium, stained with antibodies diluted in MAXbind™ Staining Medium, and washed with MAXwash™ Washing Medium. (B,E) The cells were blocked, stained, and washed with a blocking buffer containing 5% serum of the host species of the first secondary antibody, 0.2% Triton X-100, and 1× PBS. (C,F) The cells were blocked, stained, and washed with a blocking buffer containing 5% serum of the host species of the second secondary antibody, 0.2% triton X-100, and 1× PBS. Scale bars, 30 μm.



Supplementary Figure 2. Repetitive staining of only one secondary antibody. HeLa cells stained with a rabbit anti-lamin B1 primary antibody and then CF 633-conjugated donkey anti-rabbit secondary antibody multiple times. Confocal microscopy images were acquired after (A) one secondary antibody staining, (B) two consecutive secondary antibody stainings, (C) three consecutive secondary antibody stainings, and (D) four consecutive secondary antibody stainings. For each secondary antibody staining, the fresh secondary antibody solution was used. Between consecutive secondary antibody stainings, the cells were washed with MAXwash™ Washing Medium. (E) Degree of signal amplification after each secondary antibody staining (mean ± SD). Scale bars, 30 μm.



Supplementary Figure 3. Quantification of the degree of signal amplification (DSA) and signal to noise ratio (SNR) per staining round. HeLa cells stained with a rabbit anti-lamin B1 were cyclic-stained with a first secondary antibody and second secondary antibody. (A) Confocal microscopy images after 1 to 10 secondary antibody staining steps. Scale bar, 50 µm. (B) Degree of signal amplification after 1 to 10 secondary antibody staining steps (mean ± SD, average DSA of nuclear membranes profiles in 100 different locations). (C) Signal-to-noise ratio (SNR) before and after the signal amplification antibody staining steps (mean ± SD). The SNR was measured from the pre-signal amplification image shown in Figure 1C and post-signal amplification image shown in Figure 1D.

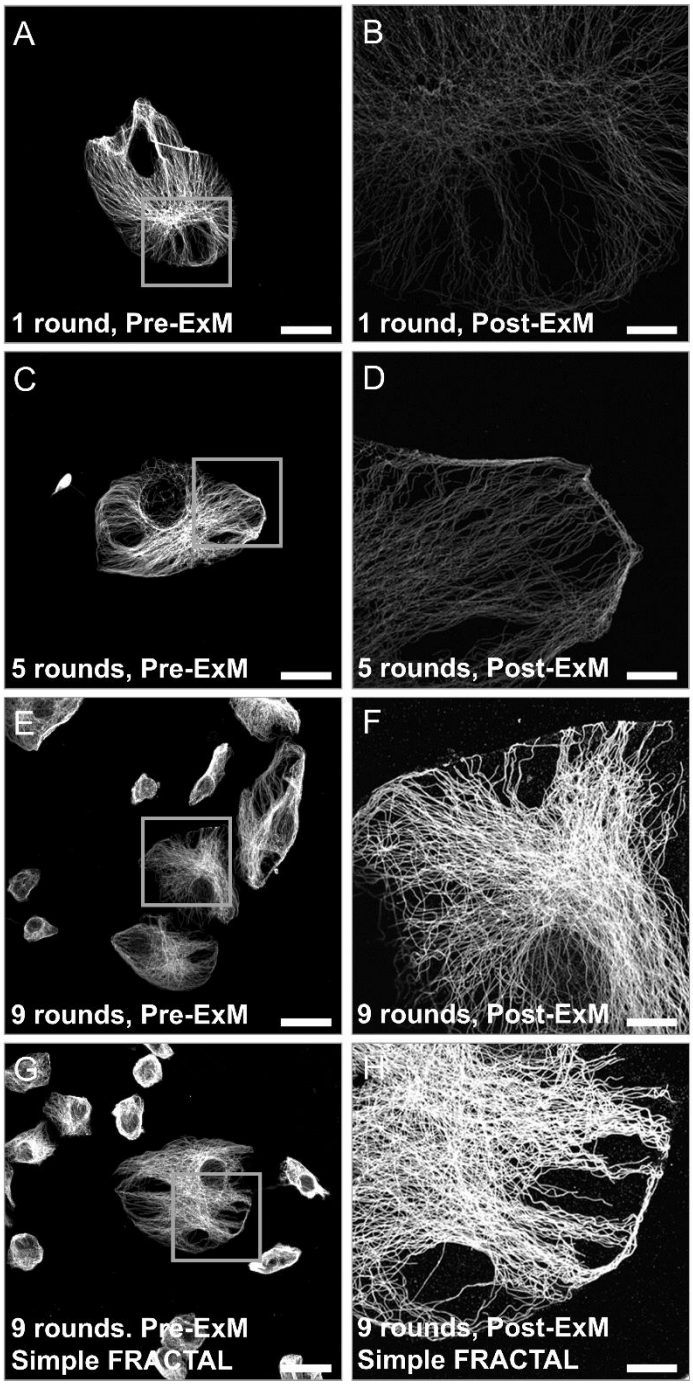


	Staining solution
①	CF 633-conjugated donkey anti-rabbit
②	Unconjugated donkey anti-rabbit
③	① + ② (1:1)
④	CF 633-conjugated rabbit anti-donkey
⑤	Unconjugated rabbit anti-donkey
⑥	④ + ⑤ (1:1)

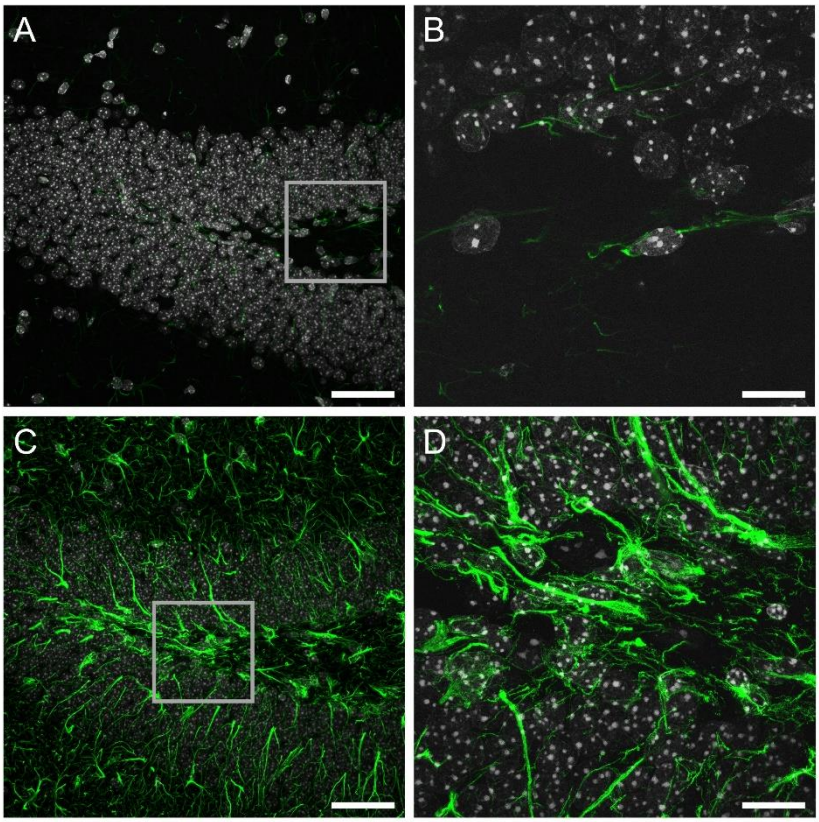
C

Degree of signal amplification			
Rounds	Regular ①+④	Simple ①+⑤	Mixed ③+⑥
1	1	1	1
2	2.07	-	1.54
3	3.24	3.20	2.45
4	4.64	-	3.24
5	5.41	4.44	3.07
6	5.00	-	3.76
7	5.91	5.72	4.11
8	6.35	-	4.77
9	7.73	6.59	4.82
10	9.32	-	6.43

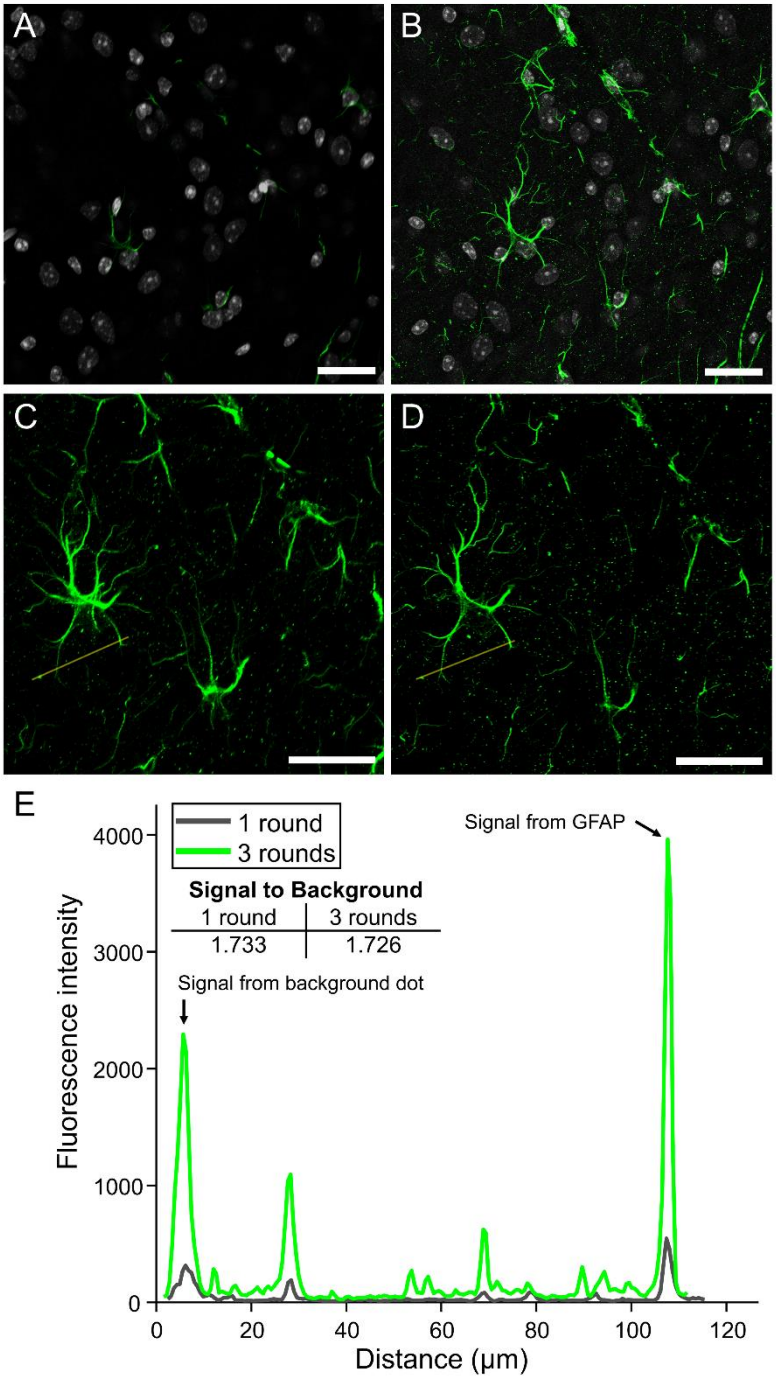
Supplementary Figure 4. Three FRACTAL techniques. (A) Schematic of three different staining methods; regular, simple, and mixed FRACTAL. (B–C) Degree of signal amplification of three different staining methods. The DSAs of simple FRACTAL were measured only after the odd-numbered staining rounds because a fluorophore-conjugated antibody was employed at the odd staining rounds, and an unconjugated antibody was utilized at the even staining rounds (mean ± SD). (C) Degree of signal amplification at each round.



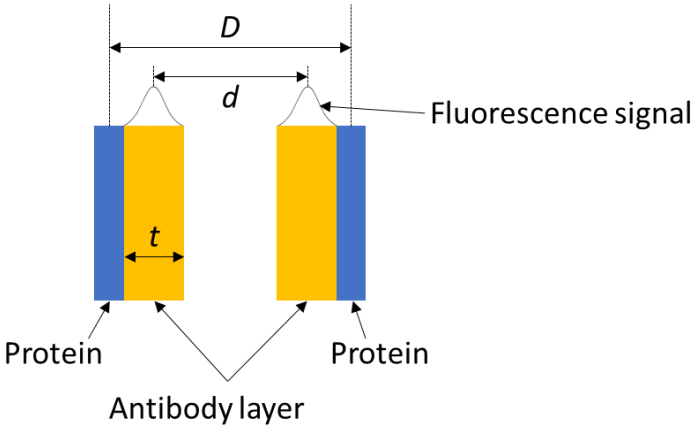
Supplementary Figure 5. ExM imaging of FRACTAL-processed HeLa cells. Pre- and post-expansion images of BS-C-1 cells stained with a rabbit anti-α-tubulin antibody and signal-amplified *via* FRACTAL. (A, C, E) Pre-expansion images after 1, 5, and 9 rounds of secondary antibody staining *via* regular FRACTAL. (G) Pre-expansion image after 9 rounds of secondary antibody staining *via* simple FRACTAL. (B, D, F, H) Post-expansion images of the images shown in A, C, E, and G. Post-expansion images shown in B, D, F, and H were acquired in the same imaging condition. Pre-expansion images shown in A, C, E, and G were acquired in different imaging conditions to clearly visualize the stained structures. Images shown in B, D, F, and H correspond to the white boxes in A, C, E, and G, respectively. Scale bars, 50 μm.



Supplementary Figure 6. ExM imaging of FRACTAL-processed mouse brain slice. (A) Confocal microscopy image of the hippocampus of a mouse brain slice, after regular antibody staining against GFAP. (B) Post-expansion image of the same brain slice shown in A. (C) Equivalent to A but signal amplification *via* regular FRACTAL. A total of four secondary antibody staining steps were performed. (D) Post-expansion image of the same brain slice shown in C. Gray: DAPI; green: GFAP. Pre-expansion images shown in A and C were acquired in the same imaging condition. Post-expansion images shown in B and D were acquired in the same imaging condition. Scale bars, 50 μm.



Supplementary Figure 7. ExM imaging of post-ExM FRACTAL-processed mouse brain slice. (A–D) Confocal microscopy images of post-expansion without or with post-ExM signal amplification. Gray: DAPI; green: GFAP. (A) Without signal amplification. (B) With signal amplification. A total of three rounds of secondary antibody staining *via* regular FRACTAL were performed. Gray: DAPI; green: GFAP. (C) Fluorescence intensity adjusted image of the same brain slice shown in A. (D) Image of the same brain slice shown in B. (E) Fluorescence intensity of line profile of C and D. Images shown in A and B were acquired with the same imaging condition. The image shown in C is into the same fluorescence intensity with the image shown in D. Scale bars, 25 μm. Scale bars of the post-expansion images were divided by the expansion factor.



Supplementary Figure 8. Schematic diagram showing two proteins and antibodies bound only to the inner surfaces of the proteins. *D*: distance between the two proteins. *d*: center-to-center distance of two fluorescence signals. *t*: the thickness of an antibody layer.

$$d = D - t, t = 8n \quad (n\text{: number of staining rounds})$$

d should be larger than the resolution of the microscope to resolve the two proteins. If the resolution of the microscope is 250 nm,

$$d = D - 8n > \frac{250}{F} \quad (F\text{: expansion factor})$$

$$D > \frac{250}{F} + 8n$$

As shown above, the minimum distance between the two proteins that can be resolved *via* ExM increases with the staining rounds. Three additional rounds would degrade the ExM resolution by 24 nm. As we assumed the worst case, which would be comparably rare, the resolution of ExM imaging of FRACTAL-processed specimens would be higher than this value. For example, if antibodies bind to both sides of the proteins, the center-to-center distance between the two fluorescence signals, each of which originates from one protein, would not deviate much from *D*. In this case, the resolution of ExM would not degrade. One solution to the degradation of resolution is the staining of expanded specimens. We have shown that FRACTAL can be applied to expanded specimens (Supplementary Figure 7). The resolution degradation of post-expansion staining can be written as follows for the antibody distribution shown in Supplementary Figure 8:

$$D > \frac{250}{F} + \frac{8n}{F}$$

As shown above, the combination of FRACTAL and post-expansion staining protocols would be an effective way to achieve both super-resolution and high signal-to-noise ratio simultaneously.

#	Host	Target	Vendor	Product Number
1	Rabbit	Bovine	Jackson ImmunoResearch	301-005-003
2		Chicken		303-005-003
3		Dog		304-005-003
4		Goat		305-005-045
5		Syrian Hamster		307-005-003
6		Horse		308-005-003
7		Human		309-005-082
8		Mouse		315-005-045
9		Rat		312-005-045
10		Sheep		313-005-045
11		Guinea Pig	Thermo Fisher	A18885
12		Hamster		A18891
13		Camelid		A16156
14		Donkey		SA1-26816
15		Cat		A18879
16		Alpaca (1)	Capralogics	PL0122
17	Goat	Bovine	Jackson ImmunoResearch	101-005-165
18		Cat		102-005-003
19		Chicken		103-005-155
20		Guinea Pig		106-005-003
21		Armenian Hamster		127-005-160
22		Syrian Hamster		107-005-142
23		Horse		108-005-003
24		Human		109-005-088
25		Mouse		115-005-166
26		Rabbit		111-005-144
27		Rat		112-005-003
28		Swine		114-005-003
29		Hamster	Thermo Fisher	31115
30		Dog		SA5-10280
31		Camelid		A16062
32		Monkey		PA1-84629
33		Pig		PA1-84097
34		Donkey (2)	Abcam	ab98817
35	Donkey	Chicken	Jackson ImmunoResearch	703-005-155
36		Goat (2)		705-005-147
37		Guinea Pig		706-005-148
38		Human		709-005-149
39		Mouse		715-005-151
40		Rabbit		711-005-152
41		Rat		712-005-153
42		Sheep		713-005-147
43	Mouse	Goat	Jackson ImmunoResearch	205-005-108
44		Human		209-005-088
45		Rabbit		211-005-109
46		Rat (3)		212-005-168
47		Bovine	Thermo Fisher	MA1-19384
48		Monkey		SA1-10653
49		Guinea Pig		MA1-80575
50	Chicken	Mouse	Thermo Fisher	SA1-72018
51		Human		SA1-72036
52		Rat		PA1-28630
54		Rabbit		SA5-10250
55		Goat		A15965
56		Bovine (4)	CliniSciences	ABIG-10
57	Alpaca	Human		609-005-213

58		Mouse	Jackson ImmunoResearch	615-005-214
59		Rabbit (1)		611-005-215
60	Sheep	Mouse	Jackson ImmunoResearch	515-005-062
61		Rat	Thermo Fisher	PA1-28637
62		Goat		SA5-10321
63		Rabbit		A16174
64	Bovine	Goat	Jackson ImmunoResearch	805-005-180
65		Human	Thermo Fisher	SA1-9501
66		Mouse		SA1-9502
67		Chicken (4)		SA1-9500
68	Rat	Mouse (3)	Jackson ImmunoResearch	415-005-166
69	Horse	Mouse	Thermo Fisher	31181

Supplementary Table 1. List of secondary antibodies in antibody vendors. Antibodies marked by the same number are two antibodies that can be used to amplify the fluorescence signal of a target protein. (1) Rabbit and alpaca, (2) donkey and goat, (3) mouse and rat, and (4) bovine and chicken.

Cell Culture Medium		
Product Name	**Vendor**	**Product Number**
Dulbecco's modified eagles' medium (DMEM)	Thermo Fisher	11995065
Minimum essential medium (MEM)	Thermo Fisher	11095114
Penicillin-streptomycin	Thermo Fisher	15140122
Sodium pyruvate	Thermo Fisher	11360070
Fetal bovine serum (FBS)	Thermo Fisher	10082147

Staining Solution		
Product Name	**Vendor**	**Product Number**
MAXblock™ Blocking Medium	Active Motif	15252
MAXbind™ Staining Medium	Active Motif	15251
MAXwash™ Washing Medium	Active Motif	15254
Normal rabbit serum (NRS)	Jackson ImmunoResearch	011-000-120
Normal donkey serum (NDS)	Jackson ImmunoResearch	017-000-121
Normal chicken serum (NCS)	Jackson ImmunoResearch	003-000-120
Normal goat serum (NGS)	Jackson ImmunoResearch	055-000-121

Antibody				
Primary/ Secondary	**Target**	**Host**	**Vendor**	**Product Number**
Primary	Lamin B1	Rabbit	Abcam	ab16048
Primary	Vimentin	Chicken	Merck Millipore	AB5733
Primary	Tubulin	Rat	Abcam	ab6160
Primary	GFAP	Rabbit	ATLAS	HPA056030
Primary	GFAP	Goat	Abcam	ab53554
Primary	Histone	Rabbit	Abcam	ab1791
Primary	α-tubulin	Rabbit	Abcam	ab18251
Secondary	Rabbit	Donkey	Jackson ImmunoResearch	711-005-152
Secondary	Donkey	Rabbit	Thermo Fisher	SA1-26816
Secondary	Chicken	Goat	Jackson ImmunoResearch	103-005-155
Secondary	Rat	Mouse	Jackson ImmunoResearch	212-005-168
Secondary	Rabbit	Goat	Thermo Fisher	A-21244 (Alexa Fluor 647-conjugated)

Fluorophore			
Product Name	**Type**	**Vendor**	**Product Number**
CF 488	NHS-ester	Biotium	92120
CF 568	NHS-ester	Biotium	92131
CF 633	NHS-ester	Biotium	92133
DAPI	-	Sigma	D9542

Fluorophore Conjugation		
Product Name	**Vendor**	**Product Number**
Sodium bicarbonate	Sigma Aldrich	S6297
NAP-5 columns	GE healthcare	17-0853-02
Amicon Ultra-0.5 Centrifugal Filter Unit	Merck Millipore	UFC510096

FFPE Sample Preparation Solution		
Product Name	**Vendor**	**Product Number**
Xylenes	Sigma Aldrich	534056
Ethyl alcohol	Sigma Aldrich	E7023
Sodium citrate tribasic dihydrate	Sigma Aldrich	C8532
Cytoskeleton Extraction Buffer		
Product Name	**Vendor**	**Product Number**
Magnesium chloride ($MgCl_2$)	Sigma Aldrich	M8266
Ethylene glycol-bis (2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA)	Sigma Aldrich	E3889
Piperazinediethanesulfonic acid (PIPES)	Sigma Aldrich	P1851
Sodium hydroxide (NaOH)	Sigma Aldrich	S8045
Triton X-100	Sigma Aldrich	T9284
Fixation Buffer		
Product Name	**Vendor**	**Product Number**
Paraformaldehyde (PFA)	Electron Microscopy Sciences	15170
Formaldehyde (FA)	Sigma Aldrich	F8775
Glutaraldehyde	Sigma Aldrich	G5882
Sodium borohydride	Sigma Aldrich	452882
Glycine	Sigma Aldrich	50046
10× PBS	Invitrogen	AM9625
Expansion Microscopy		
Product Name	**Vendor**	**Product Number**
Acryloyl-X, SE, 6-((acryloyl) amino) hexanoic Acid, Succinimidyl Ester	Thermo Fisher	A-20770
Sodium acrylate	Sigma Aldrich	408220
Acrylamide	Sigma Aldrich	A9099
N,N'-Methylenebisacrylamide	Sigma Aldrich	M7279
Sodium chloride (NaCl)	Sigma Aldrich	71376
Ammonium persulfate (APS, initiator)	Sigma Aldrich	A3678
N,N,N′,N′-Tetramethylethylenediamine (TEMID, accelerator)	Sigma Aldrich	T7024
4-Hydroxy-TEMPO (4HT, inhibitor)	Sigma Aldrich	176141
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	EDS
Trizma hydrochloride (Tris-HCl)	Sigma Aldrich	T3038
Triton X-100	Sigma Aldrich	X100
Proteinase K	New England Biolabs	P8107S
Sodium dodecyl sulfate (SDS)	Sigma Aldrich	L3771
STORM Imaging Buffer		
Product Name	**Vendor**	**Product Number**
Mercaptoethylamine (MEA)	Sigma Aldrich	30070
Glucose oxidase	Sigma Aldrich	G2133
Catalase	Sigma Aldrich	C3515

Supplementary Table 2. List of Materials used in this work.

Main Figures					
Figure	**Samples**	**A: Primary Antibody***	**B: First secondary antibody***	**C: Second secondary antibody***	**Staining***
Fig. 1(C–D)	Brain slices	Rabbit anti-GFAP	B: CF 633-conjugated donkey anti-rabbit	C: CF 633-conjugated rabbit anti-donkey	For fig. 1B: AB For fig.1C: ABCBC
Fig. 2(B–E)	Cytoskeleton extracted BS-C-1 cells	Rabbit anti-α-tubulin	B: Unconjugated donkey anti-rabbit or B': Alexa Fluor 647-conjugated goat anti-rabbit	C: Unconjugated rabbit anti-donkey	For fig. 2B and C: AB' For fig. 2D: ABCBCB' For fig. 2E: ABCBCBCBCB'
Fig.3(B–F)	HeLa cells	A: Rabbit anti-lamin B1	B: CF 633-conjugated donkey anti-rabbit	C: CF 633-conjugated rabbit anti-donkey	ABCBC
		A: Chicken anti-vimentin	B: CF 568-conjugated goat anti-chicken		AB
		A: Rat anti-tubulin	B: CF 488-conjugated mouse anti-rat		AB
Fig.3(G–K)	FFPE	A: Rabbit anti-histone	B: CF 633-conjugated donkey anti-rabbit	C: CF 633-conjugated rabbit anti-donkey	ABCBC
		B: Chicken anti-vimentin	B: CF 568-conjugated goat anti-chicken		AB
		C: Rat anti-tubulin	B: CF 488-conjugated mouse anti-rat		AB
Fig. 4(B–E)	BS-C-1 cells	A: Rabbit anti-α-tubulin	B: CF 633-conjugated donkey anti-rabbit	C: CF 633-conjugated rabbit anti-donkey	For fig. 4B and C: AB For fig. 4D and E: ABCBCBCBCB

Supplementary Figures					
Figure	**Samples**	**A: Primary antibody**	**B: First secondary antibody**	**C: Second secondary antibody**	**Notes**
Fig. S1 (A-C)	HeLa cells	Rabbit anti-lamin B1	CF 633-conjugated donkey anti-rabbit	CF 633-conjugated rabbit anti-donkey	Performance of MAXblock™ Blocking Medium and MAXbind™ Staining Medium
Fig. S1 (D-F)	HeLa cells	Chicken anti-vimentin	CF 568-conjugated goat anti-chicken	CF 568-conjugated chicken anti-goat	Performance of MAXblock™ Blocking Medium and MAXbind™ Staining Medium
Fig. S2, S3, and S4	HeLa cells	Rabbit anti-lamin B1	CF 633-conjugated donkey anti-rabbit	CF 633-conjugated rabbit anti-donkey	Quantification of the degree of signal amplification (DSA) of FRACTAL
Fig. S5	Cytoskeleton extracted BS-C-1	Rabbit anti-α-tubulin	CF 633-conjugated donkey anti-rabbit	CF 633-conjugated rabbit anti-donkey	Pre- and post-expansion microscopy imaging (ExM) of FRACTAL-processed HeLa cells
Fig. S6	Mouse brain slice	Rabbit anti-GFAP	CF 633-conjugated donkey anti-rabbit	CF 633-conjugated rabbit anti-donkey DAPI	Pre- and post-expansion microscopy imaging (ExM) of FRACTAL-processed mouse brain slice
Fig. S7	Mouse brain slice	Goat anti-GFAP	CF 568-conjugated chicken anti-goat	CF 568-conjugated goat anti-chicken	Post-expansion microscopy staining of mouse brain slice

**Supplementary Table 3. Sumary of the staining protocols. ***Characters with an underline (i.e., A, B, C) represent fluorophore-conjugated antibodies. Characters without an underline (i.e., A, B, C) represent unconjugated antibodies.

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