Expansion microscopy with conventional antibodies and fluorescent proteins

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Expansion microscopy is a technique in which fluorophores on fixed specimens are linked to a swellable polymer that is physically expanded to enable super-resolution microscopy with ordinary microscopes. We have developed and characterized new methods for linking fluorophores to the polymer that now enable expansion microscopy with conventional fluorescently labeled antibodies and fluorescent proteins. Our methods simplify the procedure and expand the palette of compatible labels, allowing rapid dissemination of the technique.

Boyden and coworkers recently introduced expansion microscopy (ExM) as a super-resolution microscopy technique that uses physical expansion of fixed specimens to allow features closer than the diffraction limit of light (~250 nm) to become resolvable in the expanded specimen. Unlike other super-resolution techniques that rely on specialized instruments, ExM is compatible with standard microscopes (for example, widefield, confocal, etc.) and is poised to make a significant impact based on its accessibility and its strong performance in thick specimens.

In the initial report on ExM, imaging with ~65-nm resolution was demonstrated in cultured cells and in brain tissue using a procedure entailing the staining of a specimen with polymer-linkable probes, growth of a swellable polymer within the specimen that links to the probes, protease digestion of the specimen, and expansion of the polymer through dialysis. The polymer-linkable probes consisted of antibodies labeled with doubly modified DNA oligonucleotides that contained a fluorophore and a methacryloyl group and were designed to become covalently incorporated into the polymer. As these DNA-labeled antibodies are custom-made and require a 1- to 2-day multi-step protocol to prepare with expensive reagents, we sought to develop methods that would allow ExM to use standard fluorophore-labeled secondary antibodies lacking DNA. We refer to these standard antibodies as conventional secondary antibodies, and to their use as conventional immunostaining. We also extended our approach to allow the direct imaging of intrinsic fluorescent protein signal in ExM.

We initially reasoned that conventional fluorescently labeled antibodies could potentially be used in ExM if a sufficient number of linkages could be formed between the antibodies and hydrogel so that protease-digested antibody fragments would remain linked to the hydrogel. Indeed, we found that a 60-min treatment of fixed and conventionally immunostained cultured cells with a 25 mM solution of the amine-reactive small molecule MA-NHS (methylenebis-N-hydroxysuccinimidyl ester) conferred excellent retention of fluorescent signal after digestion and expansion (Fig. 2a–d). Omission of the MA-NHS treatment resulted in distorted images with poor retention of fluorescence (Supplementary Fig. 1). MA-NHS was chosen here because of its resemblance to the methacryloyl group originally used in the DNA-labeled antibody probes; similar reactive groups are also used for the linking of peptides or proteins to hydrogels.

As with the original ExM report, we observed fine details in the images of expanded specimens that were hidden in images of the unexpanded specimens (Fig. 2a–d). The cross-sectional profile of expanded microtubules yielded an average Gaussian-fitted full width at half maximum (FWHM) of 79 ± 9 nm (mean ± s.d., Supplementary Fig. 2). This 79-nm width was consistent with a convolution of the double-peaked cross-sectional profile of indirectly immunolabeled microtubules measured by localization microscopy (i.e., STORM, PALM, etc.) and an estimated ~65-nm expansion-corrected lateral spatial resolution. The uniformity of expansion was remarkably good across the sample, and an analysis of distortions between corresponding pre-expansion and post-expansion confocal images showed that distortions were generally below 100 nm (root mean square distance) over length scales of up to 30 µm (Supplementary Fig. 3). A comparison of expansion fidelity using DNA-labeled secondary antibodies also yielded similar results (Supplementary Fig. 4). Note that all distances and scale bars for expanded specimens in this report have been divided by their respective measured expansion factors of 4–4.2 and that all distances and scale bars therefore refer to pre-expansion dimensions.

In a second approach, we found that treatment of conventionally immunostained cultured cells with glutaraldehyde (GA) also yielded excellent fluorescence retention after digestion (Supplementary Fig. 1). Although GA postfixation is a commonly used procedure in immunofluorescence assays, GA crosslinking is also well known for use in linking proteins or enzymes to polyacrylamide gels. Correlated pre-expansion localization microscopy and postexpansion confocal microscopy measurements using GA
treatment of immunostained cells revealed that distortions were generally below 25 nm (root mean square distance) over length scales of up to 20 µm (Supplementary Fig. 5). Microtubule cross-sectional profiles had an average Gaussian-fitted FWHM of 80 ± 7 nm (mean ± s.d., Supplementary Fig. 2), indicating a spatial resolution of ~65 nm as before. A three-color stain of an early anaphase PtK1 cell produced clear images of the mitotic spindle and distinctly resolved attachments between kinetochore fiber microtubule bundles and chromosomes with good expansion fidelity (Fig. 2e–j and Supplementary Figs. 6–8). Although the DNA stain TO-PRO-3 was quenched by the polymerization reaction, we were able to stain DNA after expansion through a brief incubation step with the dye (Online Methods). A gallery of GA-treated immunostained cells for a variety of cytoskeletal structures and subcellular organelles is shown in Supplementary Figure 9.

Cells that were conventionally immunostained and treated with either MA-NHS or GA showed 3–4× brighter signal after expansion compared with untreated cells immunostained using DNA-labeled antibodies (Supplementary Fig. 10). Although fluorescence retention postexpansion was somewhat better when DNA-labeled antibodies were used than it was with MA-NHS or GA treatment of conventional antibodies (~90% compared to ~70%, Supplementary Fig. 11), we found that pre-expansion specimens were ~4× brighter with conventional antibodies than with DNA antibodies (data not shown). This increased brightness likely resulted from specimens’ ability to conjugate more of the small fluorophore molecules (~600 g mol⁻¹) to an

Figure 2 | Confocal fluorescence images of expanded cultured cells. (a) BS-C-1 cell immunostained for tyrosinated tubulin (green) and detyrosinated tubulin (magenta) using conventional secondary antibodies and partially overlaid with corresponding pre-expansion image (top). Specimen was treated with MA-NHS after immunostain. (b–d) Zoomed-in view of boxed region in a showing corresponding pre-expansion (b) and postexpansion (c) line profiles arb., (arbitrary units). (e,f) Pre-expansion (e) and postexpansion (f) images of a dividing PtK1 cell immunostained for tubulin (green) and the kinetochore protein HEC1 (red) using conventional secondary antibodies and also stained for DNA (blue) using TO-PRO-3. Specimen was treated with GA after immunostain. (g,h) Zoomed-in view of microtubule-kinetochore attachments from boxed regions in e and f. (i,j) End-on views of boxed regions in e,f before (i) and after (j) expansion (DNA channel omitted for clarity). (k) Maximum intensity projection of a fixed BS-C-1 cell expressing the endoplasmic reticulum (ER) tag Sec61β-GFP (green) and the inner mitochondrial membrane tag mito-DsRed (blue) and immunostained against the outer mitochondrial membrane protein TOM20 using a conventional secondary antibody (red). Specimen was treated with GA after immunostain. (l) Zoomed-in view of boxed region in k showing close apposition of an ER tubule with two mitochondria. (m) Cross-sectional profile of boxed region in L arb., (arbitrary units). All distances and scale bars are in pre-expansion units. Scale bars, 2 µm (a,i–k), 500 nm (b,c,g,h,l), 5 µm (e,f).
antibody than the comparably large (~6,000 g mol⁻¹) and highly charged single-stranded oligonucleotides before compromising the antibody’s binding ability. Although we used conventional secondary antibodies prepared in-house, we found that commercially obtained secondary antibodies gave comparable results (Supplementary Fig. 12).

In cultured cells, we observed that that GA-treated specimens tolerated short digestion times (~30 min) with low distortion, while MA-treated specimens required longer digestion times to avoid distortion (~12–18 h, Supplementary Figs. 13 and 14). This observation led us to ask whether fluorescent protein signals could be retained for imaging after digestion and expansion. We found that cells treated with GA retained intrinsic fluorescent signal from fluorescent proteins (GFP, DsRed) targeted to various structures when using a ~30 min digestion time (Fig. 2k–m and Supplementary Fig. 11). The use of long digestion times (>12 h) or the omission of GA treatment resulted in little retained fluorescent protein (FP) signal (Supplementary Figs. 15 and 16). Hybrid experiments using a mixture of FP and antibody stains are straightforward (Fig. 2k–m).

The above methods extended well to brain tissue. The treatment of conventionally immunostained 100-µm-thick THY1-YFP-H mouse brain slices with MA-NHS (Fig. 3) or GA retained antibody fluorescence, although we prefer the MA-NHS treatment because treatment with GA leads to higher levels of background fluorescence (Supplementary Fig. 17)⁸. Complete, high-fidelity expansion in tissue required a lower MA-NHS concentration than in cultured cells (1 mM for 60 min), presumably due to physical differences between the specimens. We therefore advise validation (through correlative imaging before and after expansion) and possible optimization of these procedures or their variations before applying them to uncharacterized specimens that may have different properties.

We immunostained THY1-YFP-H brain slices for YFP-expressing neurons and the presynaptic and postsynaptic markers Bassoon and Homer using conventional secondary antibodies (Fig. 3a–f) and then treated these slices with MA-NHS before gelation, digestion, and expansion. Presynaptic and postsynaptic densities were well resolved and junctions between synapses and dendritic spines were clearly observable (Fig. 3a–f, Supplementary Fig. 18, and Supplementary Video 1). Over length scales of up to 30 µm, we observed distortions generally below 0.2 µm (Supplementary Fig. 19). By decreasing the digestion time for MA-treated mouse brain tissue to 1 h (rather than 12–18 h), we were able to preserve intrinsic YFP fluorescence in expanded brain tissue and we were easily able to observe dendritic spines on a neurite even using a rudimentary epifluorescence microscope equipped with a 20 × 0.45 numeric aperture (NA) air-objective lens (Fig. 3i,j). Omission of MA-NHS treatment results in very weak intrinsic YFP fluorescence levels (Supplementary Fig. 20), as pointed out by Boyden and coworkers¹.

The MA-NHS and GA-polymer-linking steps resemble the formaldehyde–acrylamide linking strategy used in the CLARITY tissue clearing procedure to link protein amines to a polyacrylamide gel, although labeling of amines on the specimen with MA-NHS and GA is over an order of magnitude faster, requiring an incubation of 60 min (or less) as opposed to one or more days in CLARITY⁹,10. While MA is almost certainly being incorporated covalently into the polymer, the linking mechanism of GA-treated specimens is less obvious. GA exists in aqueous solution as a complex equilibrium distribution of monomeric and polymeric forms that contain aldehyde and alkene groups¹¹. In principle, both aldehydes and alkene groups on GA could become covalently linked to the acrylamide polymer. Additionally, the GA polymer could become linked to the gel by entanglement with the acrylamide polymer¹² or through a combination of covalent and entanglement mechanisms. Overall, we favor MA-NHS treatment for brain tissue because of its lower background signal and GA treatment for cultured cells because of its generality with both immunolabeled specimens and fluorescent proteins. Supplementary Table 1 summarizes all stain procedures and imaging conditions used in this report.

Boyden and coworkers pointed out that not all organic fluorophores survive the polymerization step (for example, several cyanine fluorophores do not survive) and they recommended use of Alexa Fluor 488, TAMRA or Atto 565, and Atto 647N¹. To this we add that Alexa Fluor 405, Atto 488, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 568, GFP, YFP, DsRed, Hoechst 33342, and SYBR Gold also survive polymerization (Fig. 2 and Supplementary Fig. 21). Additionally, in order to avoid quenching
or bleaching by the polymerization reaction, fluorophores may be introduced postdigestion by incubating the gel with labeled streptavidin for a specimen that has been labeled with a biotin-binding fluorophore such as TO-PRO-3, etc. (Fig. 2e–j and Supplementary Fig. 21).

We have introduced and characterized new polymer-linking methods for expansion microscopy which now enable the use of conventional fluorophore-labeled antibodies and FPs. These methods should make ExM more accessible to a large and growing community of researchers applying super-resolution techniques to a wide range of biological questions. These methods improve the brightness of immunostained specimens compared with that of DNA-conjugated antibodies while making use of conventional secondary antibodies that are in many cases already available in research laboratories. Given the choice, we generally prefer the immunostaining of FPs due to its enhancement of signal brightness. However, the use of intrinsic FP signals with ExM creates flexibility in multichannel situations when compatible antibody species may not be available or when FPs are separable spectrally but not antigenically (as with CFP and YFP). The use of intrinsic FP signals may also provide advantages when antibody penetration into thick samples is limited.

Expansion microscopy is a highly attractive imaging modality owing to its compatibility with conventional microscopes and conventional probes, its robust multicolor and 3D capabilities, and its optical clearing properties for thick tissues. While the method is limited to fixed specimens whose mechanical properties do not prevent expansion, the currently achieved ~65-nm resolution is sufficient to answer a wide range of biological questions and is likely to improve with further development.

METHODS
Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
T.J.C., A.R.H., H.O., R.O.L.W., and J.C.V. designed the experiments. T.J.C., A.R.H., H.O., H.-J.K., and G.J.T. performed the experiments and analysis. T.J.C., A.R.H., and J.C.V. wrote the paper and all authors commented on the manuscript. J.C.V. supervised the project.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Reagents and reagent preparation. Unconjugated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA, USA) including donkey anti-rat (712-005-151), donkey anti-rabbit (711-005-152), donkey anti-mouse (715-005-151), and donkey anti-chicken (703-005-155). An Alexa Fluor 488 conjugated donkey anti-rat antibody (712-545-150) was purchased from Jackson Immunoresearch. Primary antibodies are listed as follows: rat anti-alpha tubulin (MA1-80017, Thermo Fisher Scientific, Waltham, MA, USA), rabbit anti-detyrosinated tubulin (ab48389, Abcam, Cambridge, MA, USA), mouse anti-HEC1 (ab3613, Abcam), rabbit anti-TOM20 (sc-11415, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-GFP (A31857, Life Technologies, Carlsbad, CA, USA), chicken anti-GFP (A10262, Thermo Fisher Scientific), rabbit anti-Homer1 (160003, Synaptic Systems, Goettingen, Germany), mouse anti-Bassoon (ab82958, Abcam; see Supplementary Table 1 for antibody concentrations). Bovine serum albumin (BSA) was purchased from Santa Cruz Biotechnology. NHS-functionalized (amine-reactive) dyes and biotin were obtained from Sigma-Aldrich, (Atto 488, Atto 565, Atto 647N, St. Louis, MO, USA) or Thermo Fisher Scientific (Alexa Fluor 405, Alexa Fluor 488, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 647, EZ-link NHS-PEG-4-Biotin). Dyes were obtained in 1 mg aliquots from Thermo Fisher Scientific (Alexa Fluor 405, Alexa Fluor 488, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 647, EZ-link NHS-PEG-4-Biotin). Dyes were purchased from Thermo (EO0491) and New England BioLabs (Madison, WI, USA) and both tested negative for mycoplasma using 4′,6-diamidino-2-phenylindole dihydrochloride. Cell lines obtained from ATCC were used without additional authentication. BS-C-1 cells were cultured in MEM (ATCC, 30-2003, Manassas, VA, USA) containing penicillin and streptomycin (PS, 15140-122, Life Tech.), 10% FBS (FB22-500, Serum Source International, Charlotte, NC, USA), and nonessential amino acids (NEAA, 11140-050, Life

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Fluorescence microscopes. Confocal microscopy was performed on a Leica SP5 inverted confocal scanning microscope at the UW Biology Imaging Core (Fig. 2 and Supplementary Figs. 2–8 and 21) using a 63 × 1.2 NA water lens (Leica, Nussloch, Germany), or an Olympus upright FV1000 (Fig. 3 and Supplementary Figs. 18 and 19) with a 25 × 1.0 NA SCALE objective. Conventional widefield epifluorescence imaging was performed on an inverted Nikon Ti-S microscope configured with a 10 × 0.25 NA air objective lens (Nikon, Melville, NY, USA), 20 × 0.45 NA air objective lens (Nikon), or a 60 × 1.2 NA water-immersion objective lens (Nikon). The widefield microscope was illuminated using a four-channel light emitting diode source (LED4D120, Thorlabs, Newton, NJ, USA) using a multiband filter set (LF405/488/532/635-A-000, Semrock, Rochester, NY, USA) and images were captured with a Zyla 5.5 sCMOS camera (Andor, Windsor, CT, USA) (Supplementary Figs. 1, 9–17, 20). Localization microscopy (Supplementary Fig. 5) was performed on a homebuilt Nikon Ti-U system configured for total internal reflection fluorescence using a Nikon CFI Plan Apo Lambda 100 × 1.45 NA objective and a 647-nm diode-pumped solid-state laser source (MPB Communications, Pointe-Claire, QC, Canada). A 405-nm solid state laser (Obis, Coherent) was used for activation to increase the rate of fluorophore blinking. Localization images were acquired on an EMCCD (iXion Ultra 897, Andor) operating at 200 frames per second. A custom-built focus lock using an objective nanopositioner (Nano F-100S, Mad City Labs, Madison, WI, USA) and a 940-nm diode laser (LP-940, Thorlabs) was used to control axial drift.

Cell culture. BS-C-1 and Ptk1 cells were obtained from ATCC and both tested negative for mycoplasma using 4’,6-diamidino-2-phenylindole dihydrochloride. Cell lines obtained from ATCC were used without additional authentication. BS-C-1 cells were cultured in EMEM (ATCC, 30-2003, Manassas, VA, USA) containing penicillin and streptomycin (PS, 15140-122, Life Tech.), 10% FBS (FB22-500, Serum Source International, Charlotte, NC, USA), and nonessential amino acids (NEAA, 11140-050, Life

Preparation of fluorophore-labeled antibodies and streptavidin. Fluorophore-conjugated antibodies or streptavidin were prepared as follows. To 40 µL of unconjugated protein (~1.3 mg mL⁻¹ IgG, or 1 mg mL⁻¹ streptavidin) was added 5 µL of aqueous 1 M sodium bicarbonate (pH ~8.3) and 1 µL of NHS-dye stock in DMSO. These reagents were allowed to react at room temperature (22 °C) for ~30 min. During the reaction, a NAP-5 size-exclusion chromatography column, for purification of labeled antibody from free dye, was equilibrated by flowing ~10 mL of PBS through each column. The ~50 µL reaction was loaded onto the column followed by flowing through and discarding 650 µL of PBS and flowing through and keeping 300 µL eluate. The eluate was characterized by absorption spectroscopy by measuring the average concentration of dye and average concentration of antibody according to the instructions provided by the dye manufacturers. Care was taken to avoid adding more than ~5% DMSO to the antibody solution to avoid disturbing the antibody in all antibody-labeling reactions. The obtained dye-to-protein ratios are listed in Supplementary Table 1. The DNA-antibody conjugate was prepared using 5’ amine modified DNA (TACGCTTAAGCCTTAAGAACCTGAC) according to the protocol described previously1 (updated protocols available at http://www.expansionmicroscopy.org). The trifunctional linker was prepared from 5’ acrydite and 3’ amine modified DNA (GTT CGG ATT CTT AGG GCG TA), reacted with a ten-fold molar excess of Atto 488 NHS for 1 h at pH 8.3, and purified by cold ethanol precipitation.
PtK1 cells were cultured in RPMI (11875-093, Life Tech.) containing 10% FBS and NEAA. Cells were maintained at 37 °C environment with 5% CO₂.

Immunostaining of cultured cells. See Supplementary Table 1 for a summary and detailed list of concentrations and reagents for the preparation of all imaged specimens.

Immunostaining of BS-C-1 cells was conducted as follows. Cells were seeded at a density of ~50,000 cells per well of a 24-well plate containing a 12 mm #1.5 coverglass and incubated overnight. Cells were optionally extracted for 30s with PEM (0.1 M PIPES pH 7, 1 mM EDTA, 1 mM MgCl₂) containing 0.5% Triton X-100 immediately before fixation. The extraction step is important for high-quality stains of cytoskeletal structures, but was not used on stains of organelle structures where treatment with detergent would likely destroy the structure (see Supplementary Table 1). Specimens were fixed for 10 min in a solution containing 3.2% paraformaldehyde and 0.1% glutaraldehyde in PEM (for microtubules) or PBS (for organelles), followed by brief washing in PBS and reduction in an aqueous solution of 10 mM sodium borohydride for 5 min. After reduction, samples were washed three times with PBS and then incubated with blocking/permeabilization buffer (PBS with 3% BSA and 0.5% Triton X-100) for 30 min. Specimens were then incubated with primary antibodies in blocking/permeabilization buffer for 45 min, washed three times with PBS, and incubated for 45 min with secondary antibodies in blocking/permeabilization buffer. After three more washes with PBS, cells were treated with either GA or MA-NHS. GA treatment consisted of a 10 min, room-temperature incubation with 0.25% GA in PBS followed by washing three times with PBS. MA-NHS treatment consisted of a 60 min, room-temperature incubation with 25 mM MA-NHS in PBS followed by washing three times with PBS. For correlative pre-expansion localization microscopy and postexpansion widefield imaging of fixed BS-C-1 cells in Supplementary Figure 5, a tertiary antibody immunostain was performed including steps for: primary rat anti-tubulin, secondary Alexa Fluor 647 mouse anti-rat, tertiary Atto 488 donkey anti-mouse antibody and finally GA treatment.

Immunostaining of PtK1 cells was conducted using a variation of the above protocol for BS-C-1 cells, but with the following differences. Cells were incubated with rat anti-tubulin and mouse anti-HEC1 primary antibodies overnight at 4 °C. After washing, cells were incubated at room temperature for 45 min with secondary antibodies consisting of donkey anti-rat secondary antibody and finally GA treatment.

Mouse brain tissue dissection and preparation. All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of Washington. Mice (strain C57BL/6) were anesthetized with isoflurane and perfused transcardially with PBS, followed by paraformaldehyde (PFA, 4% wt/vol in PBS). Brains were dissected out, postfixed in 4% PFA in PBS at 4 °C for one hour and washed in PBS. Then, the brains were sliced to 100 µm thickness using a vibrotome. All mice used in this work were between the ages of 1 and 4 months at the time of dissection. Both male and female mice were used.

Immunostaining of tissue slices. 100-µm-thick mouse brain slices were first incubated in blocking/permeabilization buffer (3% BSA and 0.1% Triton X-100 in PBS) for 6–12 h at 4 °C. The tissue was then incubated in primary antibody diluted into blocking/permeabilization buffer for at least 24 h at 4 °C and was then washed three times in blocking/permeabilization buffer (20 min each). Tissues were then incubated with secondary antibody diluted into blocking/permeabilization buffer for 24 h at 4 °C and afterwards were washed three times with PBS (20 min each). The brain slices were then either treated with 0.1% GA in PBS or 1 mM MA-NHS in PBS for 1 h at room temperature following by three washes with PBS. Tissue slices that were not immunostained (samples with fluorescent protein signal preserved) were simply treated with GA or MA-NHS. See also Supplementary Table 1 for a summary and detailed list of concentrations and reagents for the preparation of all imaged specimens.

Gelation, digestion, and expansion of cultured cell specimens. Fixed cell samples on 12 mm round coverglass were incubated in monomer solution (1 × PBS, 2 M NaCl, 2.5% (wt/wt) acrylamide, 0.15% (wt/wt) N,N'-methylenebisacrylamide, 8.625% (wt/wt) sodium acrylate) for ~1 min at room temperature before gelation. Concentrated stocks of ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) at 10% (wt/wt) in water were diluted in monomer solution to concentrations of 0.2% (wt/wt) for gelation, with the initiator (APS) added last. The gelation solution (~70 µl) was placed in a 1 mm deep, 1 cm diameter Teflon well and the coverglass was placed on top of the solution with cells face down. Gelation was allowed to proceed at room temperature for 30 min. The coverglass and gel were removed with tweezers and placed in digestion buffer (1 × TAE buffer, 0.5% Triton X-100, 0.8 M guanidine HCl) containing 8 units mL⁻¹ Proteinase K added.
freshly. Unless otherwise indicated, gels were digested at 37 °C for various amounts of time as follows: MA-treated cells were digested overnight, GA-treated cells were digested for 30 min to 1 h, and fluorescent protein samples were digested for 30 min maximum. The gels (sometimes still attached to the coverglass) were removed from digestion buffer and placed in ~50 mL DI water to expand. Water was exchanged every 30 min until expansion was complete (typically 3–4 exchanges).

Postexpansion labeling of expanded cultured cell specimens with streptavidin. Expanded cultured cell specimens initially immunostained with biotin-modified antibodies were submerged in a streptavidin solution (2 μg mL⁻¹) in PBS containing 3% BSA for 45 min. The contracted gels were then washed and re-expanded in DI water.

Gelation, digestion, and expansion of mouse tissue specimens. Tissue samples were incubated in monomer solution at 4 °C for 45 min before gelation. Tissue was gelled with the same solution as cells but with the addition of 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (4-hydroxy-TEMPO) at a concentration of 0.01% (wt/wt) from a 1% (wt/wt) stock as an inhibitor to allow complete diffusion of the monomers throughout the tissue. The glass slide with the sample and a #1.5 coverglass on top separated by spacers (one #1 coverglass) on either side of the tissue was used as a gelation chamber. The samples were allowed to gel for 2–2.5 h at 37 °C. Excess gel around the samples was removed, the glass around the samples was cut to leave the tissue on a small glass square, and the samples were placed in digestion buffer with 8 units mL⁻¹ for 45 min. The contracted gels were then washed and re-expanded in DI water.

Expanded specimen handling. Expanded gels were cut to fit on coverglass (2–4 cm edge–length rectangles) and excess water was removed and then gently placed on coverglass substrates for imaging. When possible, gels were immobilized using a small amount of cyanoacrylate glue on the periphery after wicking away excess water from the edges.

Correlative localization microscopy and ExM. Pre-expansion localization microscopy images of Alexa Fluor 647 labeled microtubules were acquired at 200 Hz for ~80,000 frames at ~2 kW cm⁻² in an oxygen-scavenging switching buffer (100 mM Tris pH 8, 10% glucose (wt/wt), 0.5 mg mL⁻¹ glucose oxidase, 40 μg mL⁻¹ catalase, and 143 mM 2-mercaptoethanol). After localization microscopy, samples were washed to remove the switching buffer, gelled, digested, and expanded as described above. During gelation, the Alexa Fluor 647 signal was destroyed; however, the Atto 488 from the tertiary antibody remained fluorescent for widefield epifluorescence imaging.

Image processing. Expanded cell culture confocal z-stacks were aligned frame by frame using an automated rigid registration routine in Mathematica in order to correct for minor lateral drift during acquisition. Mitotic spindle confocal z-stacks of PtK1 cells were processed to remove peripheral nonspecific adsorption of the HEC1 antibody as follows: A binary 3D mask of the kinetochore attachments was generated by binarizing the kinetochore channel and retaining connected-component features larger than 100 voxels and within 1 μm of the outer surface of the chromosomes. The kinetochore binary mask was then dilated by three pixels and multiplied by the original channel data. The processing was performed to clarify the maximum intensity projections in Fig. 2f,i,j, but had little effect on the individual z-sections as shown in detail in Supplementary Figure 7. Localization microscopy images were analyzed as described previously⁵. Registration of pre-expansion and postexpansion correlative images was carried out in the open-source software Elastix, using rigid (similarity) and nonrigid (B-spline) transformations to determine the expansion factor and quantify distortions. Details, including example data and processing scripts (Supplementary Analysis), are included in the Supplementary Protocol.

Reproducibility. All experiments were carried out ≥3 times including all sample preparation and analysis, except as noted below. Representative data for each experiment are shown. Experiments for Supplementary Figures 5, 10c, 11, 12, and 16b were performed only once.