Molecular resolution imaging by post-labeling expansion single-molecule localization microscopy (Ex-SMLM)

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Expansion microscopy (ExM) enables super-resolution fluorescence imaging of physically expanded biological samples with conventional microscopes. By combining ExM with single-molecule localization microscopy (SMLM) it is potentially possible to approach the resolution of electron microscopy. However, current attempts to combine both methods remained challenging because of protein and fluorophore loss during digestion or denaturation, gelation, and the incompatibility of expanded polyelectrolyte hydrogels with photoswitching buffers. Here we show that re-embedding of expanded hydrogels enables dSTORM imaging of expanded samples and demonstrate that post-labeling ExM resolves the current limitations of super-resolution microscopy. Using microtubules as a reference structure and centrioles, we demonstrate that post-labeling Ex-SMLM preserves ultrastructural details, improves the labeling efficiency and reduces the positional error arising from linking fluorophores into the gel thus paving the way for super-resolution imaging of immunolabeled endogenous proteins with true molecular resolution.
By linking a fluorophore or a protein of interest into a dense, cross-linked network of a swellable polyelectrolyte hydrogel, biological specimen can be physically expanded allowing for magnified imaging with subdiffraction-resolution on conventional microscopes. Since its introduction in 2015, expansion microscopy (ExM) has shown impressive results including the magnified visualization of pre- or postexpansion labeled proteins and RNAs with fluorescent proteins (FPs), antibodies, and oligonucleotides, respectively, in isolated organelles, cells, pathogens, tissues, and human clinical specimen. In addition, various protocols have been developed to anchor proteins or RNA into charged polyacrylamide hydrogels using 2.5% (w/w) acrylamide and 8.55% sodium acrylate with 0.15% (w/w) of the cross-linker N,N′-methylenebisacrylamide accomplishes a ~4.5x linear expansion of biological specimens. Decreasing the cross-linker concentration usually permits higher gel expansion factors of up to 10x but also increases proportionally the linkage error defined by the affinity reagent, linker and fluorophore and leads to greater gel instability. It is also possible to expand samples in series enabling gel expansion factors of 20x and higher with a demonstration of 53x expansion of microtubules. However, fluorescence imaging of such greatly enlarged samples is complicated by the dilution of fluorescent labels and dramatic increase in the physical separation between the fluorophore and its target due to the linkage error. Nevertheless, ExM with lower expansion factors enables confocal diffraction-limited fluorescence imaging with spatial resolutions comparable to that of super-resolution microscopy methods.

To further enhance the resolution, ExM has been combined with structured illumination microscopy (SIM) and stimulated emission depletion (STED) microscopy. By careful optimization of the expansion protocol ExM demonstrated that even ultrastructural details of multiprotein complexes such as centrioles can be truthfully preserved. Combining ExM with SMLM methods (Ex-SMLM) can then potentially further improve the spatial resolution to enable true molecular resolution and bridge the gap to the electron microscopy regime. However, despite these apparent advantages, attempts to combine ExM with SMLM have remained rare and unoptimized due to several challenges. There are two major determinants that control the resolution of ExM, the localization precision and the localization density. The localization precision remains unaltered by sample expansion and therefore allows achieving an improved resolution depending on the expansion factor. The localization density is arguably the more important determinant for SMLM on expanded samples. According to information theory, the required density of fluorescent probes has to be sufficiently high to satisfy the Nyquist–Shannon sampling theorem. At its most basic level, the theorem states that the mean distance between neighboring localized fluorophores (the sampling interval) must be at least twice as fine as the desired resolution. In real samples, however, the relationship between localization density and resolution is far more complex. Empirically, it seems that for a given resolution the distance between neighboring localizations should be significantly less than that indicated by a naive application of the Nyquist limit.

These considerations illustrate the challenges Ex-SMLM is confronted with. First, the fluorophore density is considerably diluted in expanded samples which often results in unclear views of biological structures and complicates SMLM data interpretation. For example, a 4x expansion in three dimensions effectively lowers the labeling density 64-fold. Second, addition of a thiol-containing phosphate-buffered saline (PBS) photoswitching buffer as required for dSTORM to a swellable polyelectrolyte hydrogel with hydrophilic ionic side groups results in substantial shrinking of the gel in the worst case down to its initial size. Finally, ExM protocols use free-radical polymerization to form polymers. However, free radicals also have the potential to react with the fluorophores which can irreversibly destroy them. Therefore, the fluorophore density will be further diluted in ExM protocols that use pre-expansion labeling and consequently reduce the structural resolution. The extent of irreversible fluorophore destruction during gelation varies across fluorophores. Unfortunately, the best suited dyes for dSTORM, the carbocyanine dyes Cy3 and Alexa Fluor 647, are almost completely destroyed during gelation. Here, post-expansion labeling approaches (post-labeling ExM) offer acceptable solutions, though they require preservation of protein epitopes during expansion.

An additional benefit of post-labeling ExM is improved epitope accessibility for antibodies and a reduction of the linkage error proportional to the expansion factor compared to pre-labeling ExM. For instance, after 4x expansion, the immunolabeling linkage error of 17.5 nm defined by the primary and secondary antibodies would reduce to 4.4 nm, which is the size of a tubulin monomer. Combining SMLM with post-labeling ExM reduces the linkage error by the expansion factor and could thus enable fluorescence imaging with molecular resolution. Here, we set out to develop post-labeling Ex-SMLM with organic fluorophores with minimal linkage error.

Results

Re-embedding of expanded samples enables Ex-SMLM in photoswitching buffer. A major problem of Ex-SMLM is the shrinking of the expanded hydrogels in photoswitching buffer due to ionic interactions between ions of the buffer and the ionic side groups of the gel. Therefore, we re-embedded expanded charged hydrogels in an uncharged polyacrylamide gel as recently introduced for ExM of RNA. We started using pre-labeling ExM in combination with standard immunostaining using unmodified primary and fluorophore labeled secondary antibodies to realize Ex-SMLM (Supplementary Fig. 1). We used microtubules as reference structure to investigate the expansion factor, spatial resolution, structural distortions, and the labeling density. Microtubules are assembled from αβ tubulin heterodimers, which stack head-to-tail into polar protofilaments with a periodicity of 8 nm, with ~13 protofilaments associating laterally in parallel to form a hollow, polar cylinder (Fig. 1a).

As previously measured by transmission electron microscopy (TEM), microtubules are hollow tubes with an outer diameter of 25 nm and 60 nm, respectively, after immunostaining with primary and secondary antibodies. This results in a linkage error defined by the size of the primary and secondary antibody of 17.5 nm (Fig. 1a).

We used the proExM protocol, in which proteins are directly anchored to the hydrogel using the succinimidyl ester of 6-((acryloyl)amino)hexanoic acid (AcX). To minimize fluorophore loss during gelation in pre-labeling ExM methods, we used the rhodamine derivative Alexa Fluor 532, which retains ~50% of its pre-gelation brightness after expansion. To prevent shrinking of the hydrogel upon addition of photoswitching buffer, expanded hydrogels were re-embedded in acrylamide for serial staining of the expanded specimen. Hydrogels were incubated twice in 10% AA, 0.15% bis-acrylamide, 0.05% APS, 0.05% TEMED in 5 mM Tris (pH 8.9) for 30 min each and subsequently transferred onto coverslips functionalized with acrydite via glass silanization to minimize lateral drift of expanded samples. After polymerization of the re-embedding gel, hydrogels were immersed in photoswitching buffer containing 100 mM mercaptoethanol (MEA) in PBS. The expansion factor was determined by comparing the post-expansion and post re-embedding fluorescence images with...
pre-expansion fluorescence images. The results showed a low distortion introduced by the re-embedding process and a reduction in gel size of ~20% from ~3.9x before to ~3.1x after re-embedding (Supplementary Figs. 2 and 3).

A caveat of imaging expanded samples is that super-resolution imaging methods, and in particular SMLM, are most effective when used on thin samples located within a few micrometers above the coverslip surface. However, expanded specimen can be easily located several tens of micrometers above the coverslip. In addition, expanded specimens are transparent because they consist largely of water. Hence, the use of oil-immersion objectives and total internal reflection fluorescence (TIRF) microscopy as used in most SMLM applications to achieve a higher signal-to-background ratio is in this case not the best choice. Therefore, we decided to use a water-immersion objective and epi-illumination in all SMLM experiments. The corresponding dSTORM images of pre-labeled expanded microtubules showed homogeneously labeled filaments with some labeling gaps reflecting fluorophore and protein loss during polymerization and enzymatic digestion, respectively (Fig. 1b, c). In addition, we imaged unexpanded microtubules by dSTORM under identical experimental conditions (Fig. 1f, g).

To examine the achieved spatial resolution, cross-sectional profiles of selected microtubule areas are often consulted. If the two-dimensional (2D) projection of the fluorescence intensity distribution measured from microtubule filaments show a bimodal distribution, the peak-to-peak distance can then be fitted with a sum of two Gaussians and used as an estimate of the spatial resolution. To ensure an objective evaluation and comparison of the spatial resolution achieved, we developed “Line Profiler”, an automated image processing software. Line Profiler automatically evaluates potential regions of interest along filamentous structures to generate cross-sectional profiles that can be fit by a sum of two Gaussians to determine the peak-to-peak distance between the sidewalls of the filamentous structure (Supplementary Fig. 4).

In order to compare the experimentally measured peak-to-peak distances of different expansion protocols, we simulated transverse profiles of unexpanded and expanded microtubules using a cylindrical distribution function to describe the hollow annular structure of microtubules (Fig. 2a and Supplementary Fig. 5) similar to the approach used recently for iterative expansion. The resulting peak-to-peak distances were used to determine the molecular expansion factor of expanded immunolabeled microtubules considering the influence of the label size on the broadening of the microtubule diameter (Supplementary Table 1 and Supplementary Note 1).
Pre-labeling Ex-SMLM. dSTORM images of unexpanded and expanded microtubules clearly showed a bimodal signal distribution along the filaments, similar to that of previous super-resolution microscopy studies (Fig. 1c, d and 1g, h). When the cross-sectional profile of unexpanded microtubules was fit with a sum of two Gaussians, the peak-to-peak distance between the sidewalls showed a mean value of 36.2 ± 5.4 nm (mean ± s.d.) analyzed over several microtubule filament segments (Fig. 1i). This value is expected for the projection of a 25 nm inner diameter cylinder that has been broadened by primary and secondary antibodies on both sides by 17.5 nm (Fig. 1a) and corresponds well to the simulated peak-to-peak distance of 32.0 nm for unexpanded microtubules (Fig. 2a and Supplementary Note 1). The mean peak-to-peak distance of proExM treated and expanded microtubules was determined to be 137.1 ± 10.1 nm (mean ± s.d.) (Fig. 1e). This value corresponds to an expansion factor of 3.1x determined from

**Fig. 2 Pre-labeling Ex-dSTORM.** a Simulated intensity profiles using a cylindrical distribution function to describe unexpanded or 3.2x expanded immunostained microtubules (labeled with IgG antibodies or DNA modified IgG antibodies pre-expansion) and resulting peak-to-peak distances of the cross-sectional profiles. b dSTORM image of expanded and re-embedded α- and β-tubulin pre-labeled with secondary Alexa Fluor 532 IgG antibodies (Al532) using the MA-NHS/GA method, i.e. antibodies are cross-linked with glutaraldehyde (GA) into the hydrogel (Antibody-Al532 (GA)). c Zoom in of white boxed region in (b). d Averaged cross-sectional profile of 8 microtubule segments with a length between 1.5–6.4 μm and 28.6 μm in total measured in 4 expanded cells. e Histogram of peak-to-peak distance distribution with normalized normal curve (red) of microtubule segments analyzed in (d) at n = 8 microtubule segments in 4 cells from 1 expansion experiment with a mean distance of 133.8 ± 13.2 nm (mean ± s.d.). f Unexpanded dSTORM image of ssDNA-Cy5 secondary antibody hybridized with Cy5 bearing oligonucleotides pre-expansion (DNA-Cy5 protocol). g Magnified view of white boxed region in (f). h Average cross-sectional profile of 7 microtubule segments with a length between 1.0–1.8 μm and 8.7 μm in total. i Histogram of peak-to-peak distances with normalized normal distribution curve (red) of the data analyzed in (h) along n = 7 microtubule segments in 2 cells from 1 experiment with a mean distance of 43.9 ± 3.7 nm (mean ± s.d.). j Expanded dSTORM image of microtubules labeled with α-tubulin and dsDNA (DNA-Al532) conjugated secondary antibodies exhibiting a methacryloyl group to crosslink the DNA with fluorophores pre-expansion into the hydrogel (original ExM trifunctional label concept). k Zoom-in of white boxed region in (j). l Average intensity profile of 26 microtubule segments with a length of 2.4–10.7 μm and 118.6 μm in total. m Histogram of peak-to-peak distances with normalized normal distribution curve (red) determined from n = 26 microtubule segments in 4 cells from 1 expanded sample showing a mean distance of 226.7 ± 15.3 nm (mean ± s.d.). n dSTORM image of α- and β-tubulin expanded according to the DNA-Cy5 protocol strategy with labels at Cy5-bearing oligonucleotides introduced post-re-embedding. o Zoom in of white boxed region in (n). p Average intensity profile of 15 microtubule segments with a length between 1.6–25.1 μm and a total length of 126.0 μm in 1 expanded sample. q Histogram of peak-to-peak distances with normalized normal distribution curve (red) determined by fitting the cross-sectional profiles analyzed in (p) along n = 22 microtubule segments in 4 cells from 1 expanded sample showing a mean distance of 201.0 ± 12.9 nm (mean ± s.d.). The small logos in the upper left corner symbolize the labeling method, e.g. pre- and post-immunolabeled with or without DNA-linker, respectively. Scale bars, 2 μm (b, f, n, p), 500 nm (c, g, k, o).
simulation of expanded microtubules pre-labeled with primary and secondary IgG antibodies (Supplementary Table 1).

We next used the post-stain linker-group functionalization method (MA-NHS/GA method) as an alternative pre-labeling Ex-SMLM method. Here, the entire sample was functionalized with polymer-linking groups after conventional immunostaining. The resulting dSTORM images showed a peak-to-peak distance between the microtubule sidewalls of 133.8 ± 13.2 nm (mean ± s.d.) (Fig. 2b–e) corresponding to a simulated expansion factor of 3.0x (Supplementary Table 1). The determined peak-to-peak distance is in good agreement with the peak-to-peak distance determined from proExM expanded microtubules (Fig. 1b–e). Variations in the measured peak-to-peak distances can be well explained by varying initial expansion factors of hydrogels which are typically in the range of ~4.0–4.5x for the used ExM gel composition. Considering a ~20% reduction in gel size caused by re-embedding of the hydrogel, an ultimate expansion factor of ~3.1–3.6x can be expected which fits well with the determined molecular expansion factors.

Next, we tested the original ExM protocol with trifunctional DNA-modified secondary antibodies, which can be labeled with dye-functionalized complementary oligonucleotides that contain an acrydite linker modification. Alternatively, antibodies can be modified with a single stranded DNA that is incorporated directly into the hydrogel. Antisense dye-labeled oligonucleotides can then be hybridized after re-embedding of the hydrogel, which enables the use of fluorophores that would not survive the radical polymerization process. Since the linkage error is mainly determined by the IgG antibodies and the 40 bases long DNA strand (Supplementary Table 2) both methods still belong to the pre-labeling Ex-SMLM method (Supplementary Fig. 1).

First, we tested the approach on unexpanded microtubules and obtained peak-to-peak distances of 43.9 ± 3.7 nm (mean ± s.d.) (Fig. 2f–i) and 37.0 ± 4.8 nm (mean ± s.d.) (Supplementary Fig. 6) for labeling with Cy5- and Alexa Fluor 532-modified oligonucleotides, respectively. These values are in good agreement with the theoretically expected value of 41.5 nm for immunolabeling with 42 bases long trifunctional oligonucleotide-modified secondary antibodies (Fig. 2a). Due to the additional modification of the secondary antibodies, the peak-to-peak distances should be a few nanometers larger than the value measured for standard immunolabeled microtubules of 36.2 ± 5.4 nm (mean ± s.d.) (Fig. 1f–i).

If the oligonucleotide-modified secondary antibodies are labeled with complementary Alexa Fluor 532-modified oligonucleotides prior to expansion, we measured a peak-to-peak distance of 226.7 ± 15.3 nm (mean ± s.d.) from dSTORM images (Fig. 2j–m). Since Cy5 does not survive gelation and Alexa Fluor 532-modified oligonucleotides, performed dSTORM imaging in photoswitching buffer and determined a slightly shorter peak-to-peak distance of 201.0 ± 9.3 nm (mean ± s.d.) (Fig. 2n–q). Both values are in excellent agreement with the theoretical peak-to-peak distance of 226.5 nm and 202 nm, respectively (Fig. 2a), simulated for 3.2x expanded microtubules taking into account the length of the 42 base pair trifunctional oligonucleotide, the position of fluorophores within the DNA strand and its spatial orientation (Supplementary Fig. 7 and Supplementary Note 1). The slightly shorter peak-to-peak distance measured in the Cy5-experiment where the dye-labeled complementary strand was hybridized after expansion can be explained most likely by coiling of the single-stranded trifunctional oligonucleotide during gelation (Supplementary Fig. 7). These results indicate that Ex-SMLM can resolve linker differences of 42 DNA base pairs (corresponding to ~14.3 nm) and, interestingly conformational differences between single and double-stranded DNA linkers.

Noteworthy is that the total size of an expanded sample is not only determined by the biomolecule of interest, e.g. microtubules, but also by the fluorescent probe, e.g. primary and secondary antibodies, used to label the biomolecule of interest. Unexpanded, microtubules labeled with primary and secondary IgG antibodies exhibit a diameter of ~60 nm with a linkage error (defined by the size of the primary and secondary antibody) of 17.5 nm. For example, for 3.3x expansion this translates into a microtubule diameter of 3.3 × 25 nm = 82.5 nm whereas the diameter of the immunolabeled microtubule is substantially broadened to ~198 nm because of the linkage error of 3.3 × 17.5 nm = 57.75 nm introduced by the primary and secondary antibody that has to be added to both sides of the microtubule (Supplementary Fig. 5). In other words, even though SMLM achieves high localization precisions, a linkage error of > 50 nm undoes much, or even all, of the gain in resolution.

**Pre- versus post-labeling Ex-SMLM.** In order to reduce the linkage error, we next explored post-labeling Ex-SMLM. It has been shown that the fluorescence signals from some genetically encoded FPs as well as conventional fluorescently labeled secondary antibodies and streptavidin that are directly anchored to the gel are at least partially preserved by proExM even when subjected to the strong nonspecific proteolytic digestion used in the original ExM protocol. Therefore, we anticipated that protein epitopes might survive the proExM protocol. To compare the labeling density of pre- and post-labeling Ex-SMLM we immunostained microtubules with Alexa Fluor 532 before and additionally after expansion. Intriguingly, combining pre- with post-labeling resulted in a substantial shortening of the average peak-to-peak distance of the sidewalls of microtubules to 79.5 ± 6.6 nm (mean ± s.d.) determined from dSTORM images (Fig. 3). We speculated that the protease digestion step may destroy a large fraction of the pre-labeled antibody complexes but to our surprise, the majority of tubulin epitopes survives this critical step. Together with the increased accessibility of tubulin epitopes for primary antibodies and primary antibody epitopes for secondary antibodies after expansion this results in peak-to-peak distances undistinguishable from solely post-labeled microtubules.

To examine more quantitatively epitope survival and increased epitope accessibilities, we simulated the cross-sectional profiles expected for pre- and post-labeled microtubules. Here we assumed a ~10-fold signal dilution for the 2D projection of the fluorescence signals of 3.2x expanded pre-labeled antibodies (Fig. 3c and Supplementary Fig. 5). Hence, the cross-sectional microtubule profiles show the superposition of the profile calculated for the 3.2x expansion of 25 nm diameter microtubules post-immunolabeled and 60 nm diameter microtubules pre-immunolabeled. The resulting superposition profile shows a peak-to-peak distance of 79.5 nm (Fig. 3e) emphasizing the advantage of post-labeling Ex-SMLM. Post-labeling Ex-SMLM using the proExM protocol provides an improved labeling efficiency and a reduced linkage error. In fact, the immunolabeling linkage error of ~58 nm for pre-labeling reduces to ~5 nm for post-labeling considering a 3.2x expansion factor and thus improves the effective achievable resolution (Supplementary Fig. 8).

Therefore, dSTORM images of Alexa Fluor 532 labeled microtubules clearly revealed the hollow cylinder of microtubules (Fig. 3c) using a water-immersion objective and epifluorescence illumination, similar to recently published results obtained by DNA-PAINT TIRF microscopy and experimental point spread function fitting. The average distance between the sidewalls of...
the xz-projection of a 6.5 µm long microtubule filament was determined to 81.2 nm (Fig. 3g–h) highlighting the high spatial resolution of pre-labeling 3D Ex-dSTORM.

Post-labeling Ex-SMLM of centrioles. Motivated by the results, we set out to explore the molecular organization of centriolar organelles by Ex-SMLM. We used isolated *Chlamydomonas* centrioles, which have a characteristic 9-fold microtubule triplet-based symmetry, forming a polarized cylinder ~500 nm long and ~220 nm wide28 (Supplementary Fig. 9). Recently, U-ExM has been developed as an extension of ExM that allows for near-native expansion of organelles and multiprotein complexes and visualization of preserved ultrastructures by optical microscopy. When combined with STED microscopy, details of the ultrastructural organization of isolated centrioles such as the 9-fold symmetry and centriolar chirality could be visualized2. Advantageously, U-ExM uses post-labeling to improve the epitope accessibility after expansion. Here, we used U-ExM treated centrioles in combination with post-labeling with Alexa Fluor 647 secondary antibodies to enable dSTORM imaging, which has previously been impossible due to shrinking of expanded hydrogels in photoswitching buffer. Therefore, samples were re-embedded and transferred onto coverslips functionalized with acrydite via glass silanization to minimize lateral drift. This allowed us to perform post-labeling 3D Ex-dSTORM on ~3x expanded centrioles (Fig. 4a–d and Supplementary Fig. 10).

Alternatively, we used the spontaneously blinking Si-rhodamine dye HMSiR29 that enables SMLM in the absence of photoswitching buffer and does thus not require re-embedding. Using double-deionized water, we achieved a molecular expansion factor of ~4x (Fig. 4d–f and Supplementary Fig. 9). Unfortunately, since the pH of double-deionized water is below 7.0, HMSiR does not exhibit optimal blinking characteristics29. Addition of PBS buffer, pH 7.4 improved the blinking characteristics of HMSiR but reduced the expansion factor to ~2x, which limits the spatial resolution of the SMLM experiments (Fig. 4d, g). In contrast to 3D dSTORM images of unexpanded centrioles (Fig. 4h) post-labeling 3D Ex-SMLM clearly visualized the centriole as a bundle of nine microtubule triplets. SMLM images of expanded isolated *Chlamydomonas* centrioles showed the 9-fold symmetry of the procentrioles (Fig. 4b, f) with tubulin diameters of ~220 nm in agreement with previous studies30. Even in side views of centrioles imaged by 3D Ex-dSTORM the neighboring microtubule triplets are clearly separated (Fig. 4c). Furthermore, 3D Ex-dSTORM allowed us to resolve ring-like sub-structures of centrioles indicating hollow cylinders of microtubule triplets (Supplementary Fig. 11). According to these results, re-embedding of the sample and dSTORM in photoswitching buffer provides currently the best Ex-SMLM performance. Since microtubule triplets separated by 15–20 nm30 are very well resolved in the expanded images post-labeling Ex-SMLM exhibits a spatial resolution that is way below 15–20 nm reaching the structural resolution required to resolve the molecular architecture of centrioles.

**Discussion**

Electron microscopy has been the only viable method able to reveal the ultrastructure of organelles and molecular complexes for decades because of the diffraction limit of optical microscopy. Super-resolution microscopy offers up to ~10x higher resolution than conventional diffraction-limited microscopy11,12. Improved super-resolution microscopy methods can now localize single emitters with a precision of a few nanometers31–33, but limitations in labeling efficiency and linkage error have thus far prevented the translation of high localization precision into sub-10-nm spatial resolution. Therefore, the spatial resolution provided by all these inventive methods is currently still too low...
to unravel the composition and molecular architecture of protein complexes or dense protein networks. Expansion microscopy (ExM) represents an alternative approach to bypass the diffraction barrier. By linking a protein of interest into a cross-linked network of a swellable polyelectrolyte hydrogel, biological specimens can be physically expanded allowing for sub-diffraction resolution imaging on conventional microscopes\textsuperscript{1–10}. However, even in combination with super-resolution microscopy techniques, spatial resolutions below \( \sim 20 \text{ nm} \) have so far proven to be very difficult to achieve by ExM\textsuperscript{16}. Here, we have shown that re-embedding of expanded hydrogels enables the use of standard photoswitching buffers and dSTORM imaging of \( \sim 3.2 \times \) expanded samples. Our results demonstrate that post-labeling ExM using the proExM protocol\textsuperscript{3} or U-ExM\textsuperscript{2} provides solutions for the two major limiting problems of improved super-resolution microscopy, the labeling efficiency and linkage error. First, as shown for microtubules, expansion of the sample increases the epitope accessibility and thus the labeling efficiency. Comparison experiments demonstrated that post-labeling outperforms pre-labeling several times in this regard (Fig. 3). Second, post-labeling ExM reduces the linkage error proportionally to the expansion factor. Hence, post-immunolabeling of 3.2x expanded microtubules reduces the linkage error from 17.5 nm\textsuperscript{22} to \( \sim 5 \text{ nm} \) (Fig. 3). Since the linkage error also influences the localization accuracy and thus the effective achievable resolution (Supplementary Figs. 8 and 9)\textsuperscript{34,35} our findings are highly relevant. Very recently\textsuperscript{36,37}, trifunctional linkers have been introduced that are inert to polymerization, digestion and denaturation, and enable direct covalent linking of target molecules and functional groups to the hydrogel. Therefore, trifunctional linkers can retain a high number of labels and fluorescence molecules available for post-expansion imaging. However, since the target molecules are labeled with primary and secondary antibodies or enzymatic tags (e.g. SNAP-tags) functionalized with the trifunctional anchor before expansion, linkage errors remain. The improved labeling efficiency of post-labeling Ex-dSTORM in combination with small (1.5 \( \times \) 2.5 nm) camelid antibodies (“nanobodies”)\textsuperscript{38,39} and 10–20x expansion factors\textsuperscript{9,10} can thus pave the way for true molecular resolution imaging of endogenous proteins with 1–5 nm spatial resolution. On the other hand, at such a small length scale, distortions of the structure may occur. To realize more homogeneous gel network structures, a new gelation method composed of tetrahedron-like monomers has been introduced\textsuperscript{40}. The new tetra-gel polymer chemistry may introduce fewer spatial distortions than earlier versions, and enable molecular resolution post-labeling Ex-dSTORM with reduced distortion. Nevertheless, already \( \sim 3x \) Ex-SMLM can resolve small linker length and conformational differences between labeling approaches as shown here for oligonucleotide-functionalized secondary antibodies (Fig. 2). In addition, we have shown that post-labeling 3D Ex-dSTORM exhibits excellent structure preservation and already
Methods

Reagents. Acetic acid (A6283, Sigma), Acrylamide (AA, 40%, A4085, Sigma), Acryloyl-X, SE, 6-((acryloyl)aminohexanoic Acid, Succhinimidyl Ester (A20770, Thermo Fisher), Agarose (A9399, Sigma), Ammonium persulfate (APS, A6789, Sigma), Bind Silane (GE17-1330-01, Sigma), Bovine Serum Albumin (BSA, A2133, Sigma), Cysteamine hydrochloride (MEA, 6500, Sigma), Dextran sulfate (D9096, Sigma), DMEM/HAM’s F12 with 1-L-glutamine (Sigma, D9062), Ethanol (absolute, ≥ 99.9%, 32205, Sigma), Ethylene diaminetetraacetic acid (EDTA, Ethylen glycol tetra(N,N,N,N-tetraacetic acid (EGTA, E7027, Sigma), F(ab’)2 fragment of goat anti rabbit IgG (A-21246, Thermo Fisher), F(ab’)2 fragment of rabbit anti mouse IgG (A-11099, Thermo Fisher), Fixation was stopped by a 7 min reduction step with freshly prepared 0.5% NaBH4 were then treated with cold K-Pipes 10 mM pH 7.2. Centrioles were then loaded in a 15 ml Corex tube with a homemade adaptor and concentrator, and spun onto a 12 mm Poly-D-lysine coated coverslip through centrifugation at 10,000 rpm. Cell wall-less Chlamydomonas reinhardtii for a 15 min wash in 0.01% Tween in PBS, and twice with PBS (1×). Cells were then fixed and permeabilized simultaneously incubating a primary fixative solution of 0.3% glutaraldehyde and 0.25% Triton X-100 in CB-buffer for 90 s and then expanded in double-deionized water. Water was exchanged several times until the maximum expansion factor of the hydrogel was reached. The expansion factor was determined by measuring the diameter of the gel using a caliper. When the expansion factor did not change within three water exchanges this factor was assumed as maximum expansion of the hydrogel.

Sample preparation. For fixation, all solutions were pre-warmed to 37 °C and fixed in PBS (1×) for 10 min followed by three washes in PBS (1×) for 10 min each.

Immunostaining of unexpanded Cos-7 cells. Cells were placed in blocking buffer (5% BSA in PBS) for 1 h and then incubated for 1 h with anti-alpha tubulin primary antibody solution (ab1825, diluted 1:500, cαtubulin = 2 µg/ml) in blocking buffer (5% BSA in PBS) for 1 h, followed by three washes in PBS (1×) for 10 min each and incubation with secondary Alexa Fluor 532 IgG antibody solution in blocking buffer (A-11002, diluted 1:200, cαtubulin = 10 µg/ml) for 1 h followed by three washes in PBS (1×) for 10 min each.

Protein Retention protocol (proExM protocol). Blocking and immunostaining were performed as described under “Immunostaining of unexpanded Cos-7 cells” incubating anti-a-tubulin antibody (ab1825, diluted 1:500, cαtubulin = 2 µg/ml) and anti-fl-tubulin (T8328, diluted 1:200, cαtubulin = 10 µg/ml) simultaneously in blocking buffer as primary antibodies and Alexa Fluor 532 IgG antibodies (A-11002 and A-11099, each diluted 1:200 to cαtubulin = 10 µg/ml) in blocking buffer as secondary antibodies. For copolymerization of amine groups into the hydrogel, cells were treated with the amine reactive agent Acryloyl X-SE (0.1 mg/ml in PBS). The agent was freshly prepared from desiccated stock aliquots kept at −20 °C, incubated overnight in a humidified chamber, and subsequently washed twice for 15 min each in PBS (1×). Hydrogel formation, Proteinase K digestion and expansion in secondary antibodies were performed as described under “ExM protocol”. After re-embedding of expanded hydrogels as described in section “Bind-silane treatment and re-embedding”, samples were labelled with α-tubulin primary antibody solution (ab1825, diluted 1:500, cαtubulin = 2 µg/ml) in 2% BSA for 3 h at 37 °C and then washed twice for 30 min each in PBS (1×). Immunostaining of unexpanded Cos-7 cells using a (ab1825, diluted 1:500, cαtubulin = 2 µg/ml) and β-tubulin (T8328, diluted 1:200, cαtubulin = 10 µg/ml) antibodies as primary antibodies and a mixture of Alexa Fluor 532 IgG secondary antibodies (A-11002 and A-11099, each diluted 1:200 to cαtubulin = 10 µg/ml) in blocking buffer. After washing with PBS (1×), cells were incubated with 0.25% GA in PBS for 15 min and washed thrice with PBS (1×). Blocking and immunostaining were performed as described under “ExM protocol”. After re-embedding on 24-mm silanized round coverslips samples were incubated overnight with Cy5 antisense oligos with a DNA concentration of 0.5 mg/ml for each oligo in hybridization buffer.

Ultrastucture expansion microscopy (U-ExM). Twelve millimeters cover glasses with isolated Chlamydomonas reinhardtii strain CW15 by centrifugation at 600g for 10 min in 50 ml conical tubes. Isolated centrioles were thawed on ice and diluted with cold K-Pipes 10 mM pH 7.2. Centrioles were then loaded in a 15 ml Corex tube with a homemade adaptor and concentrator, and spun onto a 12 mm Poly-D-lysine coated coverslip through centrifugation at 10,000g for 10 min with a JS-13.1 swinging bucket rotor (Beckman) at 4 °C. Coverslips were then processed for immunostaining and expansion microscopy.

Cell culture of mammalian cells. COS-7 monkey kidney cells (purchased from CSL Cell Line Servie GmbH) were cultured at 37 °C and 5% CO2 in DMEM/HAM’s F12 medium with L-glutamine containing FBS (10%) and penicillin/streptomycin (100 µg/ml) and tetracycline (0.1 mg/ml). 20–30,000 cells per well were seeded on round 18 mm high precision cover glasses (No 1.5) in 12-well culture plates (Techno Plastic Products, 92012) and grown for 24 h prior to fixation.

DNA label with Cy5 (DNA-Cy5 protocol). Blocking and immunostaining were performed as described under “ExM protocol” with a mixture of primary a- and β-tubulin antibodies (ab1825 diluted 1:500 with 2 µg/ml and T8328 diluted 1:200 with 10 µg/ml) and DNA conjugated secondary antibodies “Antibody B Cy5” or “Antibody C Cy5” in hybridization buffer that were then directly incorporated into the hydrogel. Hydrogel formation, Proteinase K digestion and expansion was performed as described under “ExM protocol”. After re-embedding on 24-mm silanized round coverslips samples were incubated overnight with Cy5 antisense oligos with a DNA concentration of 0.5 mg/ml for each oligo in hybridization buffer.

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1:200, $c_{\text{final}} = 10\, \mu\text{g/ml}$) diluted in 2% BSA were incubated 3 h at 37 °C followed by two washing steps in 0.01% Tween in PBS for 30 min each and two washes with PBS (1x) for 3 min. Before imaging gels were washed once more overnight in PBS (1x). For imaging of uncoupled confocal microscopes (primary antibody) anti poly-

glycylated tubulin (Adipogen, 1:500) was diluted in 5% BSA in PBS and incubated for 1 h at room temperature, washed thrice in PBS for 5 min each, followed by incubation with secondary Alexa Fluor 647 (Fab’2 antibodies (A-21246, 1:200, $c_{\text{final}} = 10\, \mu\text{g/ml}$) diluted in 2% BSA for 1 h. The samples were then washed twice in 0.01% Tween in PBS and once in PBS for 10 min each.

Re-embedding of expanded hydrogels (Re-embedding protocol). To avoid shrinking caused by dSTORM photoswitching buffer and to prevent drifting of the hydrogel during image acquisition an uncharged acrylicamide gel was crosslinked throughout the hydrogel while chemically binding it to Bind-silane treated cover glasses. Round 24-mm cover glasses (high precision) were placed in imaging buffer or staining buffer depending on the sub-

gels were then covered with 3 ml of freshly prepared Re-embedding solution (10% solution was fully evaporated. Cover glasses were then rinsed with doubly deio-

damaged. After the second incubation, samples were transferred on silanized glasses. Round 24-mm cover glasses (high precision) were sonicated successively in

Microscopes. Single-molecule localization microscopy (SMLM) image acquisition was performed on a custom-built setup with an inverted Zeiss Axio Observer Z1 (Carl Zeiss Microscopy) microscope equipped with a Definite Focus autofocus system. For excitation of different fluorescent molecules the setup provides three iBeam smart diode lasers with 405 nm (100 mW output power), 488 nm (200 mW output power) and 640 nm (150 mW output power) and a DPSS (diode pumped solid state) 532 nm laser (g532, Laserquantum). Lasers were filtered with laser clean-up filters according to the specification and focused on the back focal plane of the objective to achieve a wide field illumination. To match the aqueous refractive index of expanded samples a water-immersion objective (LD C-

Mounting and SMLM image conditions. Re-embedded hydrogels immobilized on 24-mm cover glasses were immersed in photoswitching buffer consisting of 100 mM cysteamine hydrochloride (MEA) in PBS with optimized pH (adjusted with KOH) before imaging on the uLENS. For Alexa Fluor 647 (Fab’2 antibodies (A-21246, 1:200, $c_{\text{final}} = 10\, \mu\text{g/ml}$) diluted in 2% BSA for 1 h. The samples were then washed twice in 0.01% Tween in PBS and once in PBS for 10 min each.

| Gaussian: $y = h e^{-\frac{(x-c)^2}{2b^2}} + b$ (1) |
| Bi-Gaussi: $y = h_1 e^{-\frac{(x-c_1)^2}{2b_1^2}} + h_2 e^{-\frac{(x-c_2)^2}{2b_2^2}} + b$ (2) |

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Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data that support the findings described in this study are available within the manuscript, the related supplementary information or deposited at https://doi.org/10.6084/m9.figshare.12415787.v1. Additional information is available from the corresponding authors upon reasonable request.

Code availability
The automated image processing software Line Profiler is available at https://line.profiler.readthedocs.io/en/latest/.

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Author contributions
F.U.Z., S.R., D.G., T.D.M.B., V.H., P.G., and M.S. conceived and designed the project. M.S, V.H., and P.G supervised the project. F.U.Z. performed all Ex-SMLM experiments. S.R. developed Line Profiler and analyzed the data together with F.U.Z.. D.G. provided the centriole samples. All authors wrote and revised the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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