

TOOLS

One step 4× and 12× 3D-ExM enables robust super-resolution microscopy of nanoscale cellular structures

Roshan X. Norman^{1,2,5*} , Yu-Chia Chen^{2,3*} , Emma E. Recchia^{2**} , Jonathan Loi^{1,2**} , Quincy Rosemarie² , Sydney L. Lesko² , Smit Patel² , Nathan Sherer^{2,6} , Motoki Takaku⁴ , Mark E. Burkard^{1,2,5,6} , and Aussie Suzuki^{1,2,6} 

Super-resolution microscopy has become an indispensable tool across diverse research fields, offering unprecedented insights into biological architectures with nanometer scale resolution. Compared with traditional nanometer-scale imaging methods such as electron microscopy, super-resolution microscopy offers several advantages, including the simultaneous labeling of multiple target biomolecules with high specificity and simpler sample preparation, making it accessible to most researchers. In this study, we introduce two optimized methods of super-resolution imaging: 4-fold and 12-fold 3D-isotropic and preserved Expansion Microscopy (4× and 12× 3D-ExM). 3D-ExM is a straightforward expansion microscopy technique featuring a single-step process, providing robust and reproducible 3D isotropic expansion for both 2D and 3D cell culture models. With standard confocal microscopy, 12× 3D-ExM achieves a lateral resolution of <30 nm, enabling the visualization of nanoscale structures, including chromosomes, kinetochores, nuclear pore complexes, and Epstein-Barr virus particles. These results demonstrate that 3D-ExM provides cost-effective and user-friendly super-resolution microscopy, making it highly suitable for a wide range of cell biology research, including studies on cellular and chromatin architectures.

Introduction

In recent decades, fluorescence microscopy has emerged as an essential tool for pinpointing the locations, architecture, and dynamics of proteins and genes within cells. However, the resolution of conventional fluorescence microscopy is constrained to ~250 nm laterally and 500 nm axially due to its point spread function (PSF) (Liu et al., 2018; Loi et al., 2023). This limitation means that many cellular macromolecular protein complexes and microbes, such as vertebrate kinetochores (~250 nm) (Cooke et al., 1990; Suzuki et al., 2011), microtubules (~25 nm) (Amos and Baker, 1979; Beese et al., 1987; Desai and Mitchison, 1997), nuclear pores (~120 nm) (Huang et al., 2022; von Appen et al., 2015), and viruses (~100 nm) (Louten, 2016), are smaller than the resolution limit of traditional fluorescence microscopy. To overcome this optical barrier, “super-resolution microscopy” has been developed, allowing researchers to study the structures, spatiotemporal dynamics, and functions of those nanoscale biomolecules with higher resolution (Hell and Wichmann,

1994; Neil et al., 1997; Rust et al., 2006; Valli et al., 2021). Nonetheless, this advanced technique often necessitates specialized optical equipment, specific fluorescent dyes, or computational post-processing for image reconstruction, thereby limiting its widespread application (Valli et al., 2021).

Expansion microscopy (ExM) is a cutting-edge super-resolution microscopy technique that enhances resolution by physically expanding biological specimens, eliminating the need for expensive super-resolution microscopes (Chen et al., 2015). In this method, cell cultures, organoids, and tissues are fixed and embedded into expandable hydrogel polymers. The gel-specimen composite then expands by absorbing water, resulting in improved resolution proportional to the expansion factor. The original and commonly used ExM technique achieves approximately fourfold expansion (Chen et al., 2015), theoretically reaching ~60 nm lateral resolution with conventional light microscopy. Since many biological structures are

¹Biophysics Graduate Program, University of Wisconsin-Madison, Madison, WI, USA; ²Department of Oncology, McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI, USA; ³Molecular and Cellular Pharmacology Graduate Program, University of Wisconsin-Madison, Madison, WI, USA; ⁴Department of Biomedical Science, University of North Dakota School of Medicine and Health Science, Grand Forks, ND, USA; ⁵Department of Medicine, Hematology/Oncology, University of Wisconsin-Madison, Madison, WI, USA; ⁶UW Carbone Cancer Center, University of Wisconsin-Madison, Madison, WI, USA.

*R.X. Norman and Y.-C. Chen contributed equally to this paper; **E.E. Recchia and J. Loi contributed equally to this paper. Correspondence to Aussie Suzuki: aussie.suzuki@wisc.edu; Mark E. Burkard: mburkard@wisc.edu

A. Suzuki is the lead contact.

© 2024 Norman et al. This article is distributed under the terms of an Attribution-Noncommercial-Share Alike-No Mirror Sites license for the first six months after the publication date (see <http://www.rupress.org/terms/>). After six months it is available under a Creative Commons License (Attribution-Noncommercial-Share Alike 4.0 International license, as described at <https://creativecommons.org/licenses/by-nc-sa/4.0/>).

smaller than this resolution limit, optimized ExM methods have been developed to achieve greater than fourfold expansion. One approach involves sequential fourfold expansion processes, which has been reported to achieve ~20-fold expansion (Chang et al., 2017; M'Saad and Bewersdorf, 2020). However, the iterative expansion process has several limitations, such as complicated and time-consuming sample preparation, low reproducibility of the expansion factor, and potential structural distortion. Another approach utilizes different gel chemistry, enabling ~10-fold expansion, but requires specialized equipment to remove oxygen during gel polymerization (Truckenbrodt et al., 2018). Therefore, the goal for the next generation of ExM is to achieve greater than fourfold isotropic expansion with a simple single-step process.

Here, we introduce two robust ExM methods, 4× and 12× 3D-ExM, which ensure both 3D isotropic expansion and structural preservation of biospecimens. Researchers can choose either the 4-fold or 12-fold expansion protocol based on their desired resolution, and both involve single-step sample expansion without the need for specialized instruments or chambers. We validate the 3D isotropy of 3D-ExM by measuring the area and volume of the nuclei, the largest organelle in mammalian cells, where achieving isotropic expansion has been challenging with previous ExM protocols (Büttner et al., 2020; Pernal et al., 2020). Additionally, we demonstrate that 3D-ExM resolves biological structures below the diffraction limit, including (1) the nuclear and cytoplasmic rings within a single nuclear pore complex (NPC), (2) individual viral particles of Epstein-Barr virus (EBV), (3) genomic RNA of the human immunodeficiency virus (HIV), and (4) human kinetochores during mitosis.

Results

Validation of 3D isotropic nuclear expansion by 3D-ExM

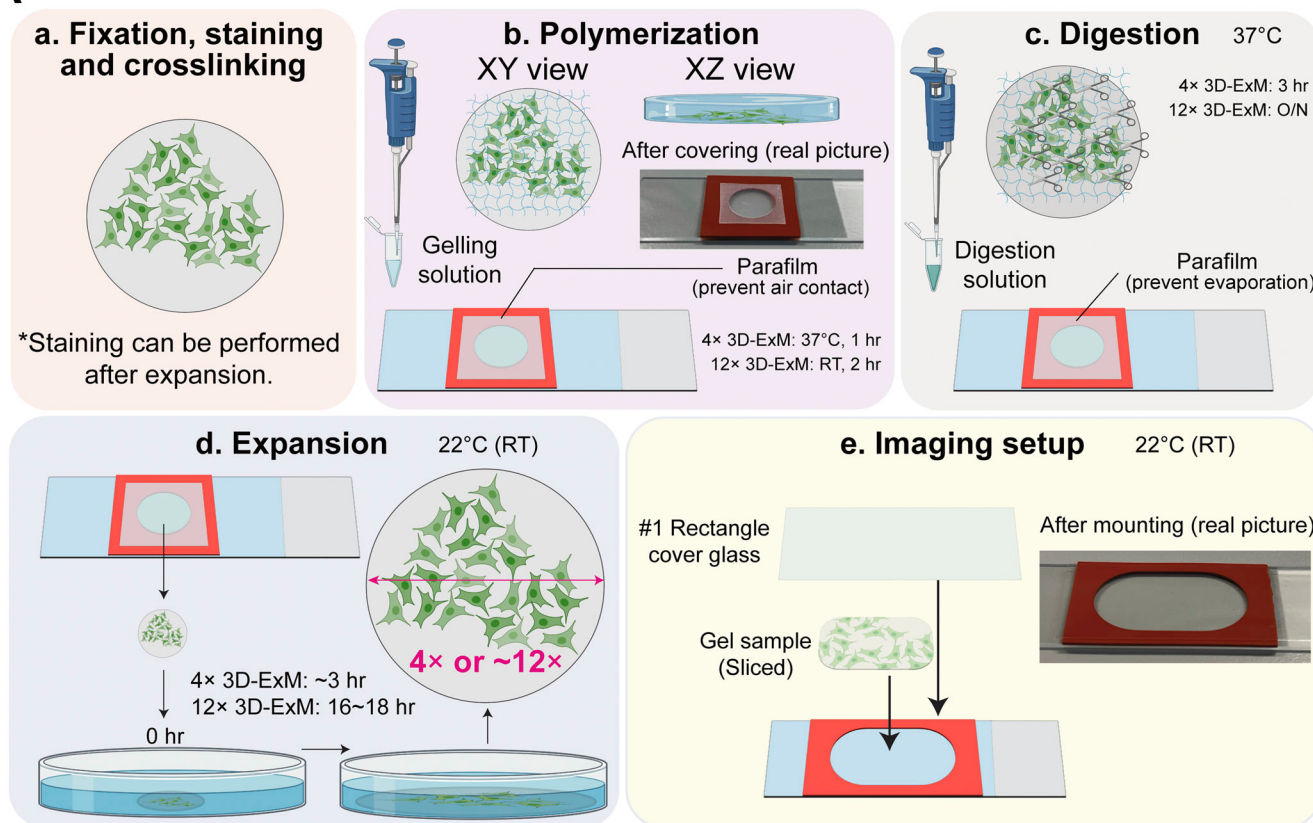
The workflow for 4× and 12× 3D-ExM is illustrated in Fig. 1 A, with a detailed protocol provided in Materials and methods. This protocol introduces several innovative steps, including the assembly of a homemade, reusable chamber for gel polymerization and imaging, designed to ensure a reproducible expansion process and minimize the drift of expanded hydrogels during imaging. The 3D-ExM approach offers researchers two distinct hydrogel recipes, enabling either fourfold (called 4× 3D-ExM) or 11–12-fold (12× 3D-ExM) expansion in a single-step process. The 4× 3D-ExM hydrogel consists of acrylamide and N,N-methylenebisacrylamide (MBAA), as used in the original ExM method (Chen et al., 2015). In contrast, the 12× 3D-ExM hydrogel is composed of N,N-dimethylacrylamide (DMAA) and sodium acrylate (SA), based on a protocol previously used for 10-fold gel expansion that required an oxygen-free environment (Truckenbrodt et al., 2018). We have developed a robust DMAA-based polymerization technique that simplifies this process by using a piece of paraffin film to minimize air contact during gel formation (Fig. 1 A). This approach enables DMAA-based hydrogel polymerization without requiring specialized equipment. We confirmed that both MBAA- and DMAA-based hydrogels expanded isometrically by ~4- and ~12-fold, respectively, in both diameter and thickness compared with the pre-expanded gels

(Fig. 1 B). Note that full expansion requires 2–3 h for the 4× hydrogel, whereas 16–18 h are needed for the 12× hydrogel.

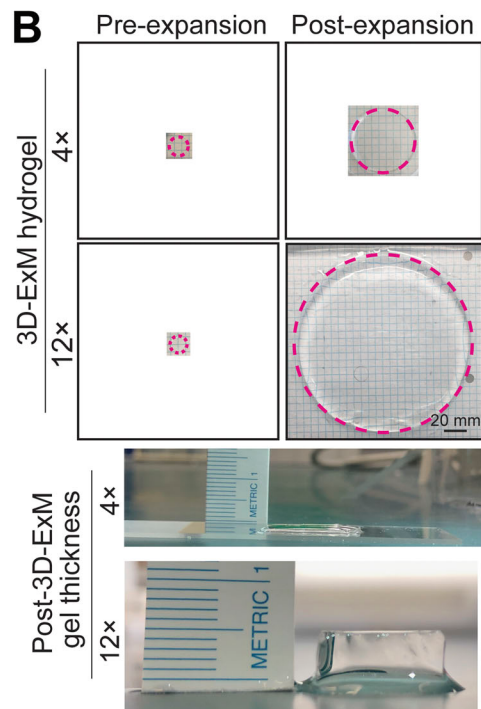
Next, we assessed the expansion isotropy of biological specimens embedded in the two types of hydrogels. Specifically, we focused on the nucleus of interphase cells, the largest and most structurally intricate organelle in mammalian cells. Previous ExM methods reported that the mammalian nucleus did not expand proportionally to the expansion factor of the gels, resulting in anisotropy even along the XY-axis (Büttner et al., 2020; Pernal et al., 2020). We measured the lateral expansion factor by averaging the lengths of the major and minor axes of the nucleus before and after expansion (Fig. S1 A). The axial expansion was determined by the volume of the nucleus through 3D rendered surface fitting (Fig. S1 B and Materials and methods). We first evaluated the expansion factor of the nucleus in 4× hydrogel and 12× hydrogel using the original crosslinking and digestion protocols of 4× ExM (Chen et al., 2015) and 10× ExM (Truckenbrodt et al., 2018) with cervical carcinoma HeLa cells and rat kangaroo PtK2 cells. Surprisingly, the nuclei of HeLa and PtK2 cells expanded only 2.8–2.9-fold in 4× ExM and 4.5–6.5-fold in 10× ExM, even though both hydrogels robustly expanded by 4- and 12-fold, respectively (Fig. 2, A and B). These findings suggest that the original ExM protocols do not achieve isotropic expansion for all cellular structures. Given that the chromatin fibers in the nucleus might restrict nuclear expansion, we treated the samples with micrococcal nuclease (MNase) before expansion. However, this did not improve the expansion of the nucleus, indicating that DNA linkage does not limit nuclear expansion (Fig. S1 C). Next, we explored whether crosslinking between proteins and hydrogel polymers restricts nuclear expansion. We tested various concentrations of Acryloyl-X (AcX), a protein crosslinking reagent commonly used in ExM (Tillberg et al., 2016; Truckenbrodt et al., 2018, 2019), and found that lower AcX concentrations yielded a higher nucleus expansion factor (Fig. S1 D). This suggested that excessive chromatin crosslinking was the primary cause of limited expansion. However, the nuclear structure became distorted at low concentrations of AcX (<30 µg/ml) (Fig. S1 D). We then tested glutaraldehyde (GA) as an alternative crosslinker. We found that 0.05–2.1% GA alone allowed for 12-fold expansion, whereas GA combined with AcX failed to support 12-fold expansion (Fig. S1 E and F). Notably, the nucleus could expand ~10-fold without any crosslinker, but the nucleus structure was significantly distorted (Fig. S1 E). These results indicate that AcX limits gel expansion and is not suitable for expansion greater than fourfold. Although GA is known to cause autofluorescence, which typically requires quenching in traditional staining protocols (Weber et al., 1978; Willingham, 1983), we observed no detectable autofluorescence after expansion within the tested range of GA concentrations (Fig. S1 E). We reasoned that the reduced autofluorescence in our ExM hydrogel-embedded biospecimens might be due to the formation of covalent bonds between GA and the gel matrix, which masked the aldehyde groups of GA.

Using our modified protocol, which features optimized crosslinking and digestion steps, we successfully achieved the expansion of the interphase nucleus of HeLa, PtK2, and human retinal pigment epithelial (RPE1) cells by ~4- and 12-fold in three

A



B



C

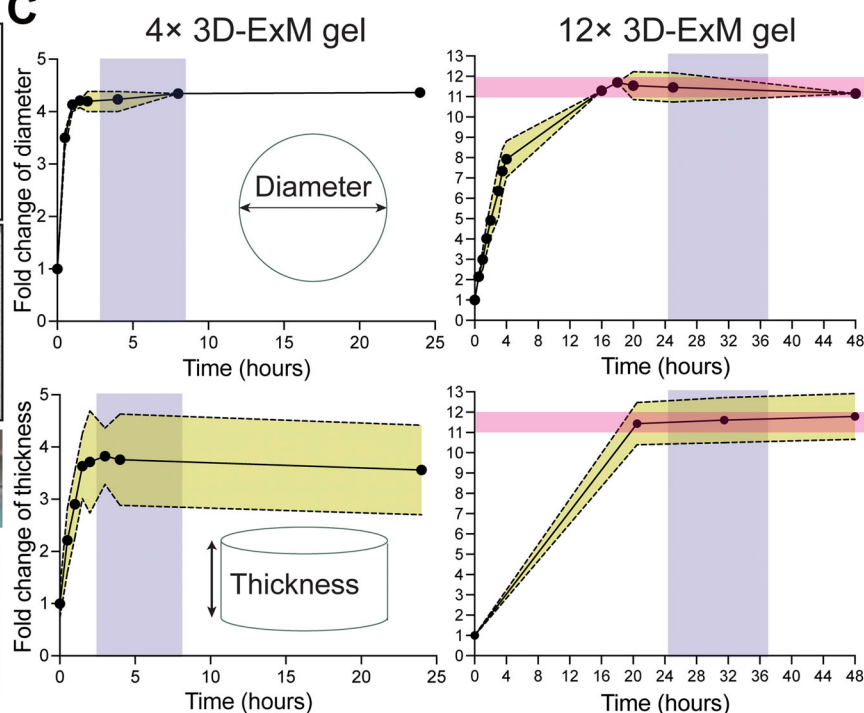
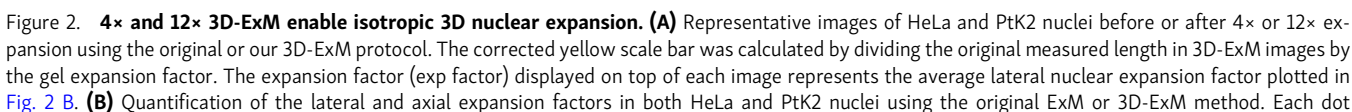


Figure 1. Validation of hydrogel expansion factors in 4× and 12× 3D-ExM methods. (A) Schematic diagram of the protocol of 3D-ExM. Detailed procedure is in the Materials and methods section. (B) Representative images of hydrogels before and after 4× or 12× expansion. The top view images show the diameter of the hydrogel. The side-view images show the thickness of the hydrogel. (C) Quantification of the diameter and thickness of 4× and 12× hydrogels at different time points after immersion in water. The yellow region indicates the standard deviation. $n = 18$ and 9 independent experiments for 4× and 12× 3D-ExM, respectively. The purple region indicates the time used for imaging in this study. 12× 3D-ExM gels reproducibly achieved 11- to 12-fold expansion in 3D (pink region).



represents a single nucleus. L: lateral, A: axial. $n = 48, 103, 62, 58, 53, 52, 42, 34, 41, 41, 46, 43, 68, 68, 45, 40$ cells (from left to right) pooled from two to three color-coded biological replicates. The P value was calculated using Welch's *t*-test (two-tailed). **** indicates $P < 0.0001$. **(C)** Left: representative images of nuclei of RPE1 cells before and after 12× 3D-ExM. Right: quantification of the lateral and axial expansion factors in RPE1 nuclei using 12× 3D-ExM. $n = 44$ and 33 cells (from left to right) pooled from three color-coded biological replicates. **(D)** The 3D view of a RPE1 nucleus. Both XZ and YZ views are shown. DAPI was used for pre-ExM imaging and DRAQ5 was used for post-ExM imaging. 60× water objective (NA = 1.20) was used for imaging. All data are shown as the mean $\pm$ s.d.

dimensions (Fig. 2, A–D). Notably, the expanded interphase nuclei preserved typical heterochromatin domains, which are evident as regions with high DNA dye signal intensity (Fig. 2 D). These results demonstrate that our 3D-ExM methods can consistently and isotropically expand nuclei within the 2D cell monolayer.

Validation of isotropic expansion by correlative pre- and post-3D-ExM

To further validate isotropic expansion in 4× and 12× 3D-ExM, we imaged the same cells before and after applying 4× or 12× 3D-ExM under identical imaging conditions (Fig. 3, A–D). Initially, we imaged DAPI-stained RPE1 cells with a 20× water objective (NA = 0.95) prior to expansion. After measuring the gel expansion factor, we re-stained the expanded gel with DAPI and imaged the identical cells using the same 20× water objective. We calculated the expansion factor based on the lengths of the major and minor axes, as well as the 2D area at the best focal plane for the same cell, both before and after expansion (see Materials and methods). In both 4× and 12× 3D-ExM, the interphase nucleus expanded proportionally to the gel expansion factor, confirming equal and isotropic expansion between specimens and hydrogels. We observed that while the overall nuclear shape and the global DAPI-staining pattern were preserved following both 4× and 12× 3D-ExM, the DAPI staining pattern exhibited subtle differences between pre- and post-3D-ExM. We proposed two potential explanations for this observation. First, the expansion process may make chromatin more open, leading to increased accessibility for DAPI and subtly altering the staining pattern compared to pre-ExM imaging. Alternatively, DAPI molecules that bound to DNA during the pre-ExM staining may have undergone photobleaching during pre-ExM imaging or 3D-ExM processes. These photobleached molecules could remain associated with the chromatin, thereby limiting the availability of binding sites for new DAPI molecules during post-expansion staining.

12× 3D-ExM enables expansion of 3D organoid culture model

We investigated whether 3D-ExM could be applied to more complex 3D organoid culture models. Human organoids derived from the MCF-7 breast cancer cell line and primary breast tumor cells were generated, and the expansion factor of their nuclei size was determined following 12× 3D-ExM (Fig. 4, A and B; and Fig. S2, A and B). The nuclei within the organoids were expanded by ~12-fold, as measured by the quantification of their volume and surface area. Notably, 12× 3D-ExM greatly improved the axial resolution, allowing for the clear identification of individual nuclei within patient-derived organoids (Fig. S2 A). Collectively, we concluded that our 3D-ExM method consistently

and isotropically expands nuclei in both 2D cell monolayers and 3D organoid culture models.

Validation of achievable resolution of 12× 3D-ExM using cellular rulers

In theory, 4× and 12× 3D-ExM could achieve ~60 and ~20 nm lateral resolution, respectively, using regular confocal microscopy (based on the ~250 nm lateral resolution of confocal microscopy). To assess the actual performance of 3D-ExM in terms of achievable resolution limits, we first measured the diameters of microtubules (~25 nm) (Weber et al., 1978), a common “cellular ruler,” in interphase PtK2 cells using both confocal and STED microscopy. Fig. 5 A presents example confocal images of PtK2 cells with fluorescently labeled DNA and microtubules using the same size of field of view (FOV) before and after expansion (4× and 12× 3D-ExM). We employed line intensity scan analysis to measure the microtubule diameter. As expected, without expansion, the microtubule diameter was ~250 nm with regular confocal microscopy and ~130 nm with STED microscopy, respectively (Fig. 5 C and Fig. S3 A). Using 4× 3D-ExM combined with regular confocal or STED microscopy, the microtubule diameter measured 80 and 60 nm, respectively (Fig. 5 C and Fig. S3 A). However, with 12× 3D-ExM, both confocal and STED microscopy yielded a consistent microtubule diameter of ~28 nm, demonstrating that 12× 3D-ExM combined with regular confocal microscopy can achieve sub-30 nm lateral resolution (Fig. 5, B and C; and Fig. S3 A). Consistent with these findings, 12× 3D-ExM enabled the resolution of the complex 2D and 3D microtubule networks within the interphase cytoplasm (Fig. S3 B). It is noteworthy that the slight difference in the measured microtubule diameter between electron micrographs (25 nm) and 12× 3D-ExM (28 nm) may result from the additional size of the antibody used in our experiments to label alpha-tubulin.

To further validate the resolution achieved by 12× 3D-ExM, we imaged Nup107 and Elys, components of the NPC, also commonly used to evaluate the resolution of imaging techniques (Huang et al., 2022; Strambio-De-Castillia et al., 2010; von Appen et al., 2015) (Fig. S3, C and D). Nup107 is a crucial structural component of both the cytoplasmic and nuclear rings of the NPC, with an inter-ring distance of ~61 nm based on previous cryo-EM data (von Appen et al., 2015) (Fig. 5 D). Elys, a nucleoporin, is exclusively located at the nuclear ring of the NPC (Huang et al., 2022). Nuclear pores (NPs) were identified as low-electron density regions at the nuclear periphery via transmission electron microscopy (TEM) (Fig. 5 E). Using 12× 3D-ExM, we observed similar features with relatively low DNA signal levels at the nuclear periphery, overlapping with NP proteins (Fig. 5 E and Fig. S3, C and D). Notably, while Elys appeared as a single dot, Nup107 presented as two foci at each NP,

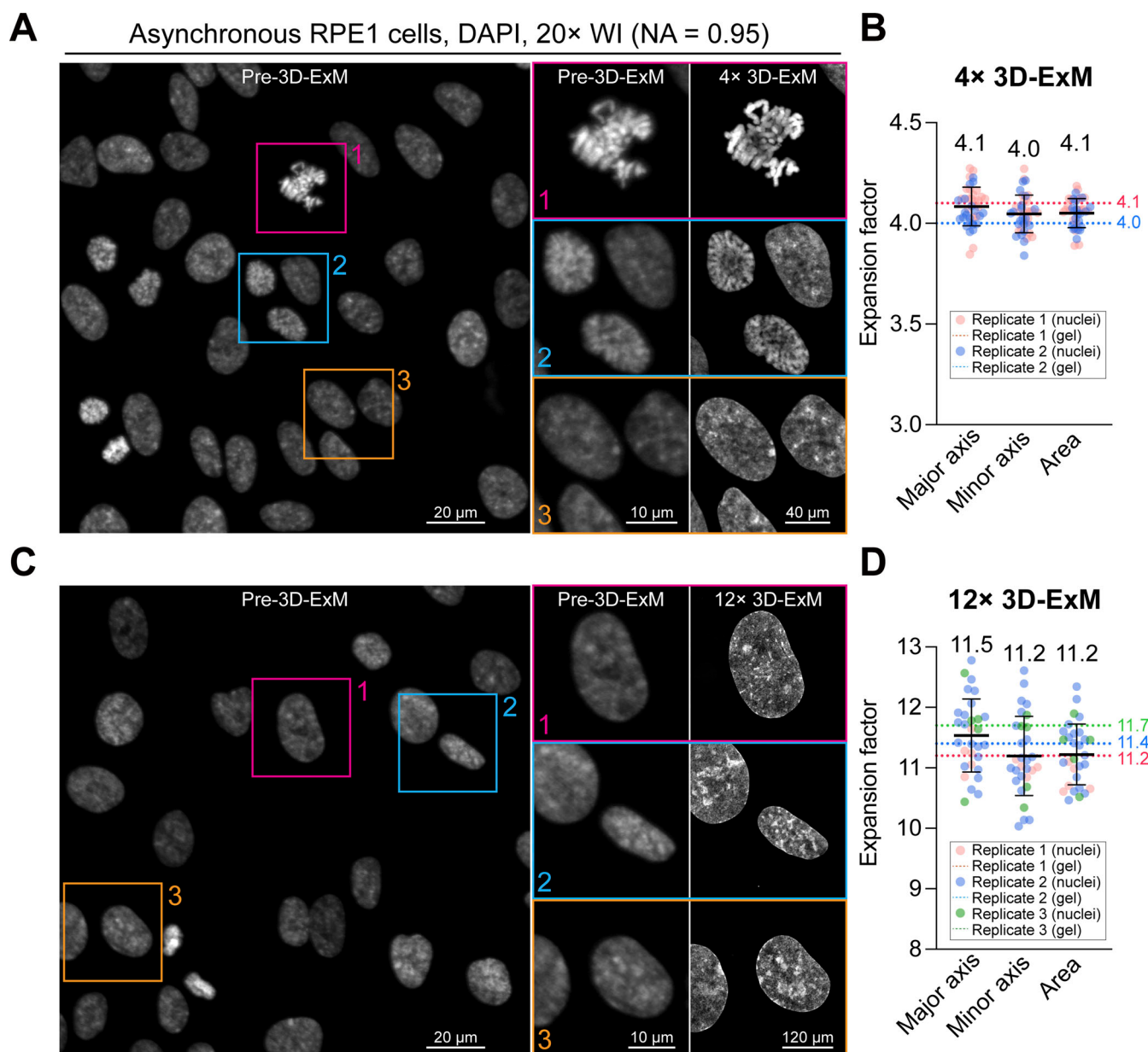


Figure 3. Validation of isotropic nuclear expansion factors through correlative pre- and post-3D-ExM. (A) Representative images of DAPI-stained RPE1 nuclei before and after 4× 3D-ExM of the same cells. Left: a pre-3D-ExM image. Three example regions of cells are highlighted by bounding boxes of different colors. Right: a direct comparison of the digitally enlarged images (pre-3D-ExM) with the corresponding 4× 3D-ExM images of the same cells. Note that both pre- and post-3D-ExM images were captured under identical imaging conditions (20× WI, NA = 0.95). (B) Quantification of the expansion factor of the same RPE1 nuclei based on their major axis length, minor axis length, and the 2D area before and after 4× 3D-ExM. The expansion factor of the area was determined by taking the square root of the ratio of the post-3D-ExM nuclear area to the pre-3D-ExM nuclear area. Each dot represents a single nucleus. $n = 40$ cells pooled from two color-coded biological replicates. The horizontal dotted line indicates the gel expansion factor in each experiment. The mean expansion factor is shown above each dot plot. (C) Representative images of DAPI-stained RPE1 nuclei before and after 12× 3D-ExM. Left: a pre-3D-ExM image. Three example regions of cells are highlighted by bounding boxes of different colors. Right: a direct comparison of the digitally enlarged images (pre-3D-ExM) with the corresponding 12× 3D-ExM images of the same cells. Note that both pre- and post-3D-ExM images were captured under identical imaging conditions (20× WI, NA = 0.95). (D) Quantification of the expansion factor of the same RPE1 nuclei based on their major axis length, minor axis length, and the 2D area before and after 12× 3D-ExM. Each dot represents a single nucleus. $n = 30$ cells pooled from three color-coded biological replicates. The horizontal dotted line indicates the gel expansion factor in each experiment. The mean expansion factor is shown above each dot plot. All data are shown as the mean $\pm$ s.d.

corresponding to the cytoplasmic and nuclear rings of the NPC (Fig. 5 F). The average corrected distance between Nup107 foci at a single NP was ~ 67 nm, consistent with cryo-EM measurements (von Appen et al., 2015). These results confirm that 12× 3D-ExM readily resolves the two pools of Nup107, ~ 60 nm

apart within a single NPC, demonstrating that the lateral resolution achieved by 12× 3D-ExM with standard confocal microscopy is significantly better than 60 nm (Fig. 5 G). Like scanning electron microscopy, 12× 3D-ExM visualizes NPs at the nuclear surface (Fig. 5 H). Nearly all NPs at the nuclear

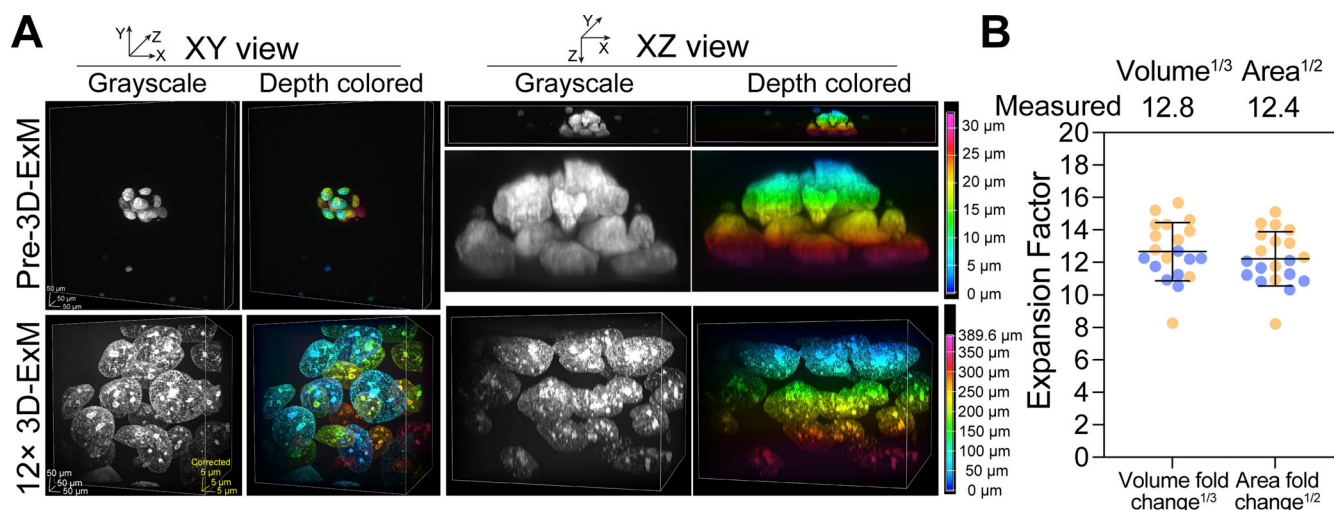


Figure 4. 12× 3D-ExM enables isotropic expansion of 3D organoid cultures. (A) Representative 3D images of human organoids acquired before and after 12× 3D-ExM. DNA was stained with DRAQ5. The depth of objects in the image are color-coded. Note that the organoids shown in pre- and post-3D ExM were distinct and not identical organoids. 20× water objective (NA = 0.95) was used for imaging. (B) Quantification of the expansion factor of the volume and surface area of individual nuclei within the organoids. Expansion factors of the volume and surface area of the nucleus after 12× 3D-ExM were calculated by the fold change in the cube root of the volume and the square root of the surface area, respectively. Results are shown as the mean ± s.d. Each dot represents a single nucleus. *n* = 20 cells pooled from two color-coded biological replicates.

surface, observed as low-intensity holes in the DNA labeling, were colocalized with Nup107 signals (Fig. 5 H and Fig. S3 E). The corrected diameter and circumference of NPs were ~120 and ~380 nm, respectively (Fig. 5 I), consistent with the measurements from prior cryo-EM research (von Appen et al., 2015). Based on the surface area and NP density, an interphase nucleus of RPE1 cells is estimated to have ~4,300 NPs (Fig. 5, J and K; and Fig. S3 F), within the range of previous estimates for human nuclei (Capelson and Hetzer, 2009; Ori et al., 2013). In summary, 12× 3D-ExM achieves the predicted image resolution with isotropic expansion for both cytoplasmic and nuclear proteins.

Determination of cellular protein and genomic architecture by 4× and 12× 3D-ExM

We investigated whether 3D-ExM could be employed to discern cellular protein complexes and genomic architecture that are too small to study using conventional fluorescence microscopy. We visualized individual virions of Epstein-Barr virus (EBV), a double-stranded DNA virus and ubiquitous human pathogen belonging to the Herpesviridae (Frappier, 2021; Sugden, 2014). Previous EM research has determined that the diameter of EBV virions ranges from 100 to 220 nm (Kieff et al., 1982; Odumade et al., 2011; Ogembo et al., 2015; Yin et al., 2018). We utilized the well-characterized iD98/HR1 cell line, an EBV-positive cell line engineered to conditionally enter the lytic stage of EBV's life cycle when EBV virions are produced (Chiu et al., 2013; Glaser and Rapp, 1972) (See Materials and methods). To visualize individual EBV virions, we fluorescently labeled the viral glycoprotein 350 (GP350), the most abundant glycoprotein on the EBV envelope (Fig. 6 A) (Germi et al., 2012). Without 3D-ExM, we observed GP350 signals in lytic cells, but could not resolve individual EBV virions (Fig. S4 A). In contrast, 4× 3D-ExM revealed individual EBV virions, as GP350 foci overlapped with

DNA puncta, indicative of encapsidated EBV genomes (Fig. 6 A). These results showed that 4× 3D-ExM combined with regular confocal microscopy is capable of resolving a single EBV virion, thus approaching the 60-nm theoretical resolution limit for fourfold expansion. Remarkably, 12× 3D-ExM resolved the viral envelope as a ring-like structure surrounding a DNA dot signal, representing the encapsidated EBV genome. In 12× 3D-ExM, the outer and inner diameter of EBV virions labeled by GP350 were 162 and 76 nm respectively, after correction by the expansion factor (Fig. 6 A), aligning with previous EM data (Nanbo et al., 2018). These findings demonstrate that 12× 3D-ExM has a comparable resolution to EM, which is sufficient to examine viral architecture (Fig. 6 A and Video 1).

We next investigated whether 12× 3D-ExM preserved RNA and enabled its detection. To this end, we utilized single-molecule fluorescence in situ hybridization (smFISH) to visualize human immunodeficiency virus-1 (HIV-1) unspliced RNA (Becker et al., 2017), transcribed from the integration sites within the nucleus containing the wild-type HIV-1 genome (Fig. S4 B). This custom RNA FISH probe, specific for the gag-pol open reading frame (Knoener et al., 2021), successfully visualized the HIV-1 genome integration site in both pre- and post-12× 3D-ExM (Fig. S4, B and C). HIV-1 unspliced RNAs are packaged as genome dimers with the viral Gag structural protein during assembly at plasma membrane sites (Fig. 6 B) (Chen et al., 2016; Keane and Summers, 2016). We performed RNA FISH coupled with Gag immunofluorescence to detect dimerized genomes associated with Gag in virus particles. Our finding revealed that virus particles shed from HIV-1-infected HeLa cells exhibited two fluorescent RNA signal peaks, co-localizing with the Gag signals (Fig. 6 B and Fig. S4 D). This suggests that 12× 3D-ExM can detect both a monomer and a dimer conformation of the HIV-1 unspliced RNA. These results demonstrate that 12× 3D-ExM

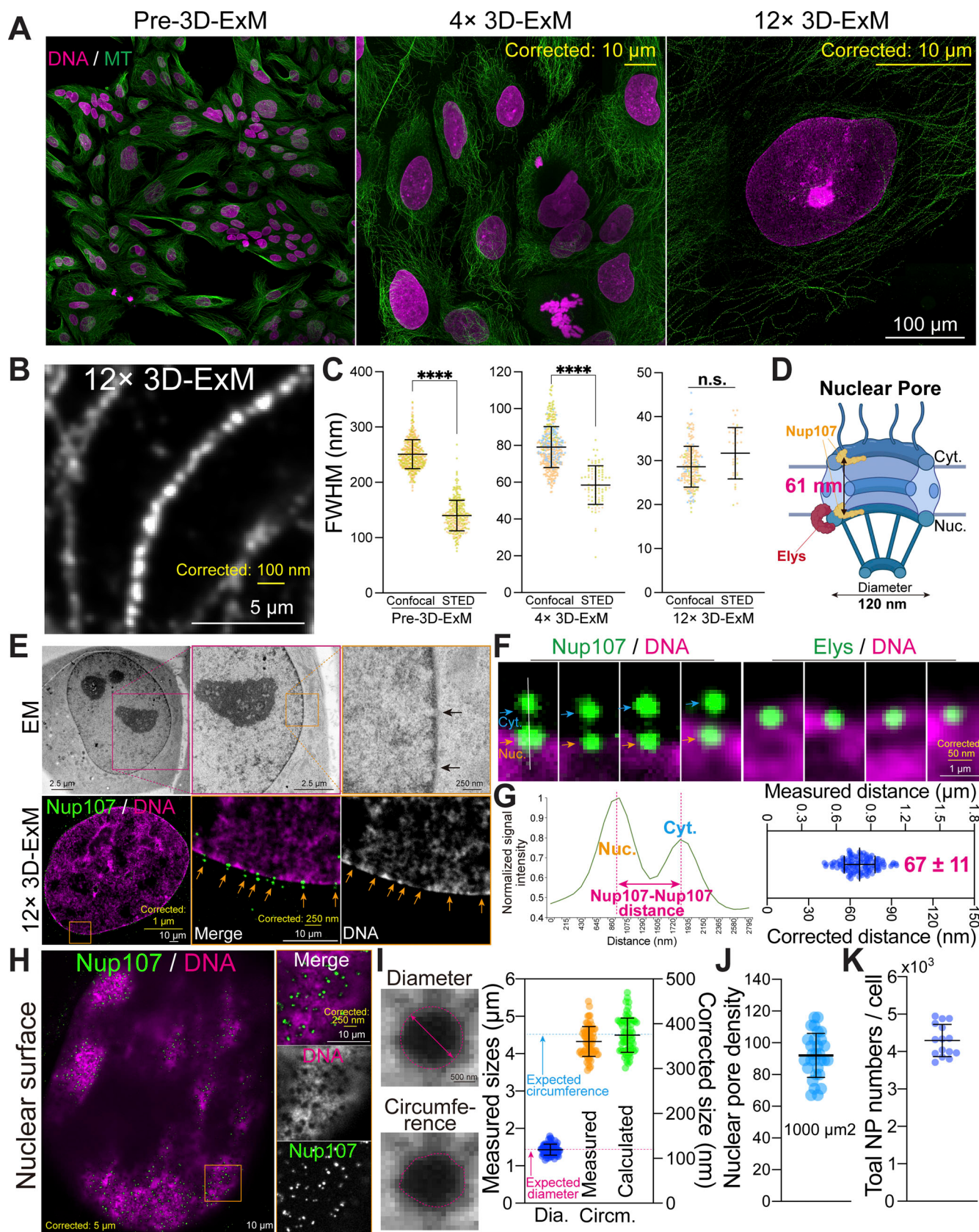


Figure 5. Validation of 3D-ExM resolution using cellular rulers. (A) Representative images of Ptk2 cells stained with tubulin (green) and DNA (magenta) acquired before expansion (pre-3D-ExM) or after expansion (4× and 12× 3D-ExM). 60× water objective (NA = 1.20) was used for imaging. Multiple full FOV 12× 3D-ExM images were combined to create a composite image of a single cell. (B) A zoom-in representative 12× 3D-ExM confocal image of microtubule filaments

in PtK2 cells. **(C)** Quantification of full width at half maximum (FWHM) of microtubules in images acquired before or after 4× or 12× 3D-ExM. Both the conventional confocal microscope and STED microscope were used in this experiment and quantification. $n = 251, 140, 221, 155, 150, 36$ cells pooled from two to three color-coded biological replicates (from left to right). The P value was calculated using Welch's *t*-test (two-tailed). **** indicates $P < 0.0001$. **(D)** Schematic diagram of the structure of NPC created by BioRender. NP proteins Nup107 and Elys are labeled with yellow and red, respectively. **(E)** The EM images (upper row) and 12× 3D-ExM images (lower row) of the nucleus and NPs at the edge of the nucleus are shown in the right rows. DNA was stained with DRAQ5. **(F)** The representative 12× 3D-ExM images of Nup107 and Elys at the edge of a nucleus. **(G)** Left: an example Nup107 intensity profile at a single NP. Right: the average measured and corrected distance between two Nup107 intensity peaks in the line scan. $n = 117$ pooled from two biological replicates. **(H)** Representative image of the surface of RPE1 nuclei stained with Nup107 and DRAQ5. The black regions shown in the DNA channel are nuclear pores that colocalize with the Nup107 punctate signals. **(I)** Left: example images of the measurement of diameter and circumference of NPs. Right: quantification of the measured or corrected values of diameter and circumference of the NPs. $n = 70$ NPs pooled from two biological replicates. The calculated circumferences were derived from the diameters of the NPs, while the measured circumferences were obtained independently of their diameters. **(J)** Quantification of the number of NPs per unit area ($1,000 \mu\text{m}^2$, equivalent to $\sim 7 \mu\text{m}^2$ after correction by the expansion factor). $n = 30$ cells pooled from two biological replicates. **(K)** Quantification of the estimated total number of NPs per cell. Each dot represents a single cell. $n = 15$ cells pooled from two biological replicates. All data are shown as the mean $\pm$ s.d.

effectively preserves RNA integrity and enables super-resolution imaging of RNA using smFISH. Furthermore, we employed 4× 3D-ExM to visualize the intricate structure of centrioles (Fig. S5, A–C). Centrioles are a pair of cylindrical structures (mother centriole and procentriole) arranged perpendicular to each other (Bettencourt-Dias and Glover, 2007). Unlike the procentriole, the mother centriole is distinguished by the presence of a distal appendage at its distal end (Bettencourt-Dias and Glover, 2007). To examine these structures, we labeled CEP164 (a marker for the distal appendages) and acetylated-tubulin (a marker for the centriole wall) in cold-treated RPE1 cells. The 4× 3D-ExM revealed the cartwheel structure of the mother centriole, showcasing a universal ninefold radial symmetry from the top view. Additionally, the distal appendage was observed as a protrusion at one end of

the mother centriole from the side view (Fig. S5 A). The measured diameter of the distal appendage ring and the length of centrioles obtained through 4× 3D-ExM were consistent with previous measurements using other super-resolution imaging methods (Bowler et al., 2019; Chang et al., 2023; Sahabandu et al., 2019) (Fig. S5, B and C).

The kinetochore is a macromolecular protein complex that assembles on centromeres, serving as a microtubule attachment site and orchestrating chromosome movements during mitosis (Musacchio and Desai, 2017). The human kinetochore displays a plate-like structure, ~ 300 nm long and 50 nm thick, as observed by EM (DeLuca et al., 2005; Dong et al., 2007). Due to the lateral PSF and 3D orientation of kinetochores, they usually appear as puncta in conventional fluorescence microscopy rather than rectangular plates (Fig. 7, A and B, top, CENP-C and Plk1 serve as

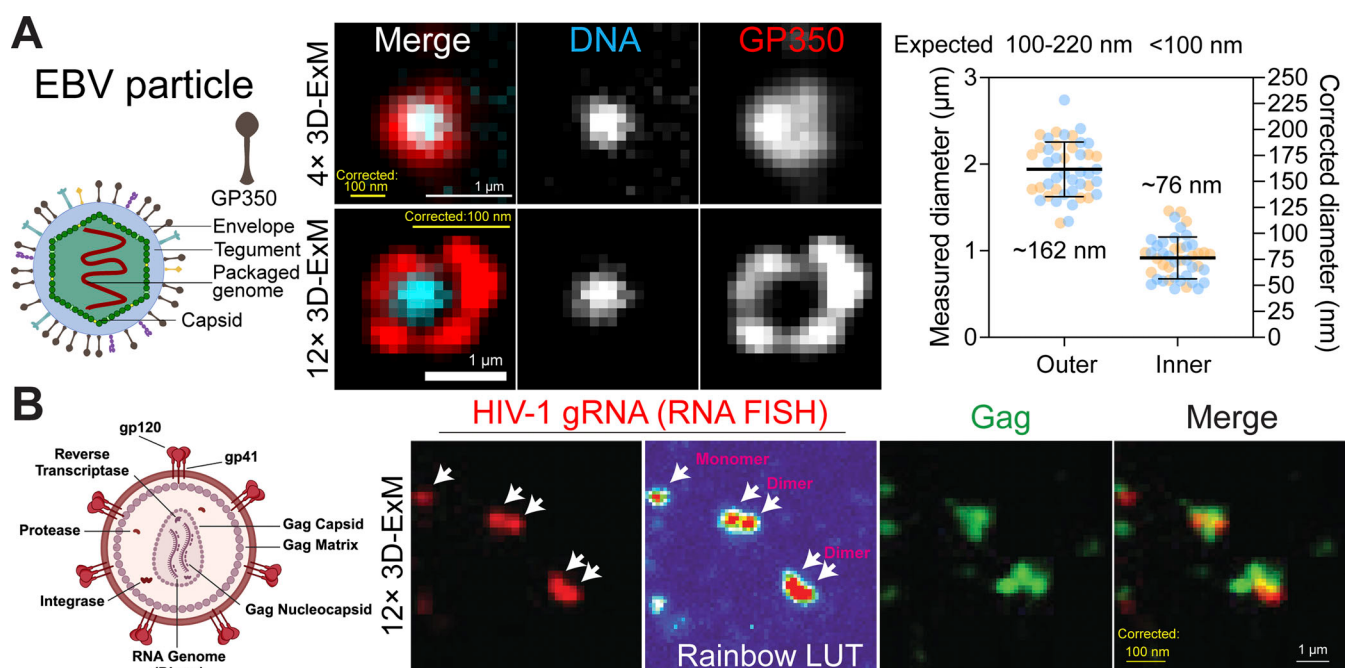


Figure 6. Application of 4× and 12× 3D-ExM in visualizing viruses in host cells. **(A)** Left: schematic diagram showing the structure of EBV. GP350 is a viral envelope glycoprotein. Middle: representative 4× and 12× 3D-ExM images of EBV virions. Right: quantification of the measured and corrected diameter of EBV virions. Results are shown as the mean $\pm$ s.d. $n = 44$ virus particles pooled from two color-coded biological replicates. DNA was labeled with DRAQ5. **(B)** Left: schematic diagram showing the structure of HIV. Right: representative 12× 3D-ExM images of HIV gRNA and Gag proteins. HIV gRNA was labeled using specific FISH probes.

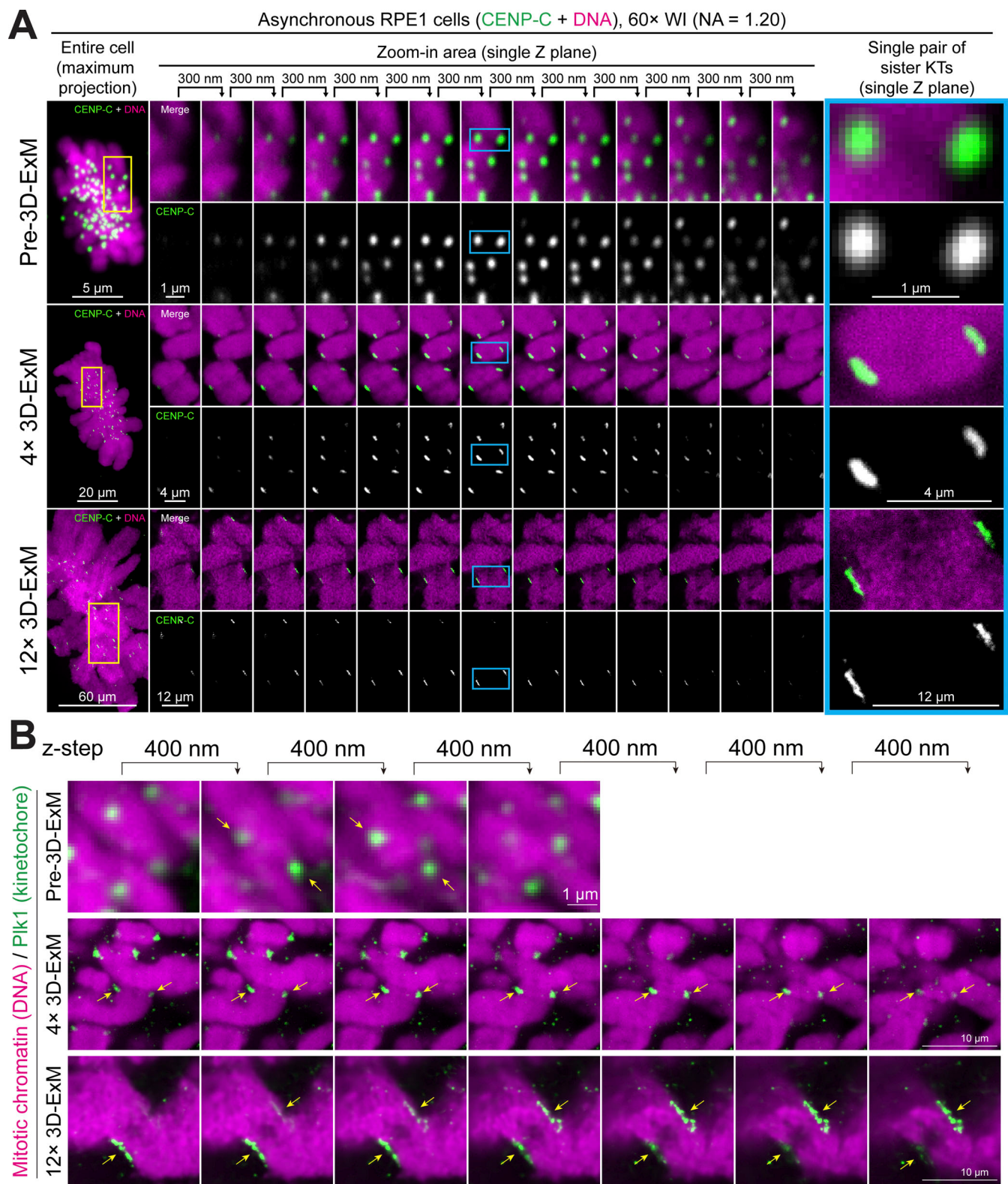


Figure 7. Application of 4× and 12× 3D-ExM in visualizing kinetochores. (A) The serial z-stack images of kinetochores labeled with CENP-C at metaphase in RPE1 cells were acquired before expansion (pre-3D-ExM) and after expansion (4× and 12× 3D-ExM) with a step size of 300 nm. The blue bounding box indicates a pair of sister kinetochores that assemble on the same chromosome. (B) The serial z-stack images of mitotic RPE1 cells stained with anti-Plk1 antibodies and DNA dye (DRAQ5) were acquired before expansion (pre-ExM) and after expansion (4× and 12× 3D-ExM) with a step size of 400 nm. Arrows indicate a pair of sister kinetochores that assemble on the same chromosome. 60× water objective (NA = 1.20) was used for imaging.

kinetochore markers). While the lateral PSF does not interfere when the objects are larger than 250 nm in length, the axial PSF significantly overestimates the size, even if the axial length exceeds 1 μm (Loi et al., 2023). We imaged metaphase cells in both pre- and post-3D-ExM using the same imaging condition with a 60 $\times$ water objective (NA = 1.20). Supporting this, CENP-C kinetochore signals, prior to 3D-ExM, appeared in ~ 11 separate optical sections spaced at 300 nm depth, overestimating the size to $>3 \mu\text{m}$ axial length compared with the ~ 300 nm size determined by EM (Fig. 7 A, top). Using 4 $\times$ 3D-ExM, kinetochores appeared more rectangular, though still not matching the plate-like structures seen in EM (DeLuca et al., 2005; Dong et al., 2007). The axial length was also significantly overestimated, showing $\sim 3 \mu\text{m}$ optically (corrected: ~ 750 nm), in 4 $\times$ 3D-ExM (Fig. 7 A, middle). These discrepancies are likely due to the combined effects of axial PSF and the 3D orientation of kinetochores (Loi et al., 2023). In contrast, kinetochores in 12 $\times$ 3D-ExM exhibited clear plate-like structures with the expected axial length of $\sim 3 \mu\text{m}$ optically (corrected: ~ 250 nm) (Fig. 7 A, bottom). Consistent with the kinetochore structure labeled by CENP-C, Plk1 distribution at kinetochores displayed plate-like structures in 12 $\times$ 3D-ExM with a significant improvement in both lateral and axial resolution as compared with 4 $\times$ 3D-ExM (Fig. 7 B). The 12 $\times$ 3D-ExM technique enables visualization of the kinetochore-microtubule interface with a resolution previously unattainable through light microscopy (Fig. S5 D). Notably, the distribution of CENP-C in both 4 $\times$ and 12 $\times$ 3D-ExM images at metaphase closely resembled structures previously observed through immunoelectron microscopy (Marshall et al., 2008; Suzuki et al., 2011). However, we did not observe the distinct bipartite architecture of CENP-C reported by a recent study (Sacristan et al., 2024). Instead, our results showed that CENP-C appeared in a continuous, plate-like distribution in both 4 $\times$ and 12 $\times$ 3D-ExM. This difference may be attributed to differences in the protocols used. Sacristan et al. employed the original 4 $\times$ ExM protocol, which, based on our analysis of interphase nuclei, resulted in insufficient crosslinking and digestion for the nucleus to undergo isotropic expansion (Fig. 2 B). It is plausible that condensin and cohesin complexes are compartmentalized at the inner centromere, and incomplete crosslinking and/or digestion enhance the visualization of these compartments in ExM images. Alternatively, our fixation protocol for immunofluorescence in 3D-ExM did not involve any pre-extraction or permeabilization in the fixation solution, whereas the referenced study performed pre-extraction with 0.5% Triton X-100 prior to fixation. Given that previous studies have demonstrated the effect of pre-extraction or permeabilization during fixation on the intrakinetochore stretch (Magidson et al., 2016; Suzuki et al., 2018), we cannot rule out the possibility that the difference in fixation protocols influences the kinetochore structures observed by ExM. Further investigation is required to elucidate these structural differences at the kinetochore. Collectively, our data illustrate that 12 $\times$ 3D-ExM provides sufficient resolution to minimize the effects of axial PSF and the 3D orientation of cellular architectures below the diffraction limit, such as kinetochores, allowing for accurate 2D/3D measurements of these nanoscale cellular structures.

Practical applications of 12 $\times$ 3D-ExM

We demonstrated the capabilities of 12 $\times$ 3D-ExM through two practical examples. Aneuploidy, characterized by the gain or loss of chromosomes due to mitotic errors, is a hallmark of cancer and correlates with therapeutic outcomes (Sansregret et al., 2018). To accurately determine the karyotype of cells and evaluate the pattern and extent of aneuploidy, the chromosome spread technique is commonly employed. This method involves swelling and bursting cells in a hypotonic solution. However, this process frequently results in chromosome overlap, which can lead to erroneous chromosome identification and quantification (Remya et al., 2019). Recent studies have attempted to map chromosomes in intact cells using serial block-face scanning electron microscopy (SEM) (Booth et al., 2016; Chen et al., 2017). However, these efforts have only successfully identified a subset of chromosomes in Colcemid-treated cells and were unable to perform quantifications in multiple cells due to the time-consuming nature of the method. 12 $\times$ 3D-ExM is expected to achieve resolution comparable with or better than serial block-SEM (Booth et al., 2016), with the added advantages of simple sample preparation and specific labeling of target molecules. To ascertain if the complete karyotype of PtK2 cells could be accurately identified without using chromosome spreads, we utilized immunofluorescence to label kinetochores and DNA in asynchronous PtK2 cells, which possess 14 chromosomes (Lorenz and Ainsworth, 1972). While PtK2 cells possess fewer chromosomes than human cells, high-resolution confocal microscopy using a 60 $\times$ oil objective (NA = 1.40) was insufficient to determine the karyotype and identify individual chromosomes in an intact metaphase cell (Fig. S5 E). In contrast, the 12 $\times$ 3D-ExM method enabled us to distinctly visualize each chromosome within an intact metaphase cell (Fig. 8 A and Video 2). Furthermore, we successfully identified all chromosomes in these cells by analyzing chromosome size and relative kinetochore location along the chromosome (Fig. 8 A and Fig. S5, F-H). Using the same approach, we determined the aneuploidy status of a single intact cell (Fig. 8 A; and Videos 3 and 4). We identified aneuploid cells by simply counting the number of chromosomes in an intact mitotic cell and pinpointed which chromosomes were gained or lost without the need for chromosome spreading, FISH, or averaging multiple cell quantifications. These results demonstrate that 12 $\times$ 3D-ExM can precisely determine the complete karyotype at a single-cell resolution. Additionally, this method enables the exploration of the spatial arrangement of chromosomes at any stage of mitosis, which is important for understanding the mechanisms underlying chromosomal instability (CIN) (Klaasen et al., 2022).

Chromosome bridges, a type of mitotic error, are defined by chromatin linkages between sister chromatids during anaphase, often arising from DNA damage and cohesion defects (Finardi et al., 2020). These bridges frequently result in the formation of micronuclei, which can lead to chromothripsis and CIN (Leibowitz et al., 2015). An example 12 $\times$ 3D-ExM image of a HeLa cell with a chromosome bridge is shown in Fig. 8 B. Unexpectedly, this 12 $\times$ 3D-ExM image revealed that the chromosome bridge could be comprised of two intertwined sister chromatids (Fig. 8 B). This finding underscores the capability of 12 $\times$ 3D-ExM

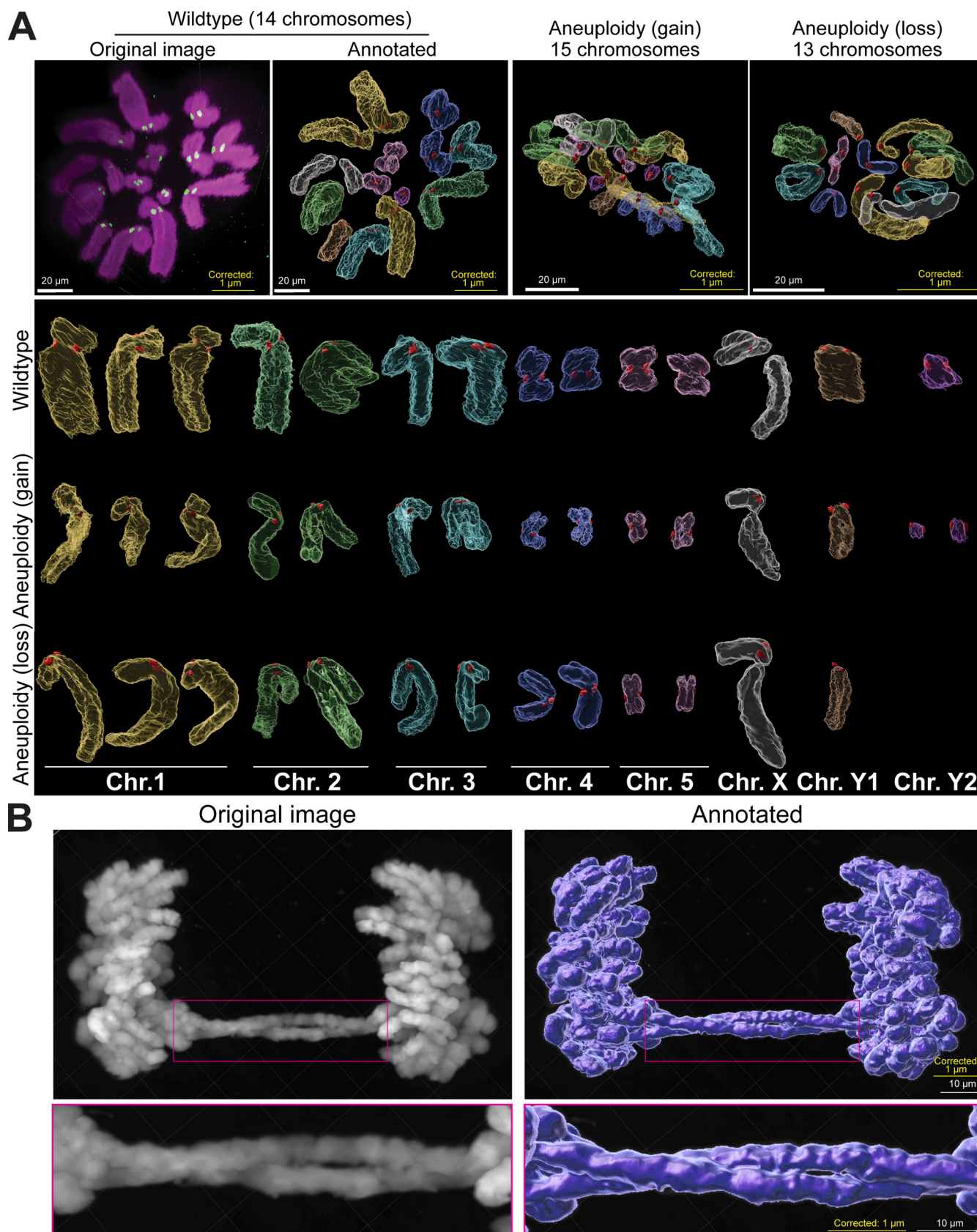


Figure 8. **Application of 12 $\times$ 3D-ExM in chromosome identification and examination of the detailed architecture of chromosomes.** (A) Top: example 12 $\times$ 3D-ExM images of three mitotic PtK2 cells with one wild-type and two aneuploid karyotypes (gain or loss of Chr. Y2). Bottom: the entire karyotype of individual chromosomes for each cell. 3D rendering of mitotic chromosomes was performed using Imaris (Andor). DNA was stained with DRAQ5. (B) An example 12 $\times$ 3D-ExM image of an anaphase HeLa cell with the chromosome bridge composed of two twisted chromosome strands. DNA was stained by DAPI. 60 $\times$ oil objective (NA = 1.40) was used for this imaging.

to uncover previously invisible chromosomal architecture, potentially leading to novel discoveries.

Nuclear staining is one of the most widely used techniques in cell biology. We demonstrated the performance of various DNA dyes for 3D-ExM (Fig. S6 and Materials and methods). The tested DNA dyes included DAPI, Hoechst 33342, Nuclear Green LCS1, SYTOX Green, propidium iodide (PI), and DRAQ5, covering a broad range of excitation wavelengths. Both DAPI and Hoechst showed clear, DNA-specific staining in both 4× and 12× 3D-ExM. While PI and DRAQ5 also provided distinct DNA staining, they exhibited a higher cytoplasmic background compared with DAPI and Hoechst, likely due to their affinity for RNA. This cytoplasmic background can be minimized by treating samples with RNase prior to crosslinking. Although LCS1 and SYTOX Green were detectable in both 4× and 12× expanded gels, both dyes showed rapid photobleaching compared with the others. The DNA dyes tested showed no detectable bleed-through or cross-excitation under our imaging conditions, offering researchers the flexibility to choose dyes tailored to their microscope setup or specific experimental requirements.

Discussion

Fluorescence microscopy was invented over 100 years ago. Since then, both fluorescence microscopes and fluorophores, such as dyes and proteins, have been continuously improved, expanding their applications across many research fields. To address the challenges of studying subcellular structures below the resolution limit of conventional light microscopy, various super-resolution imaging technologies have been developed. However, these methods often require expensive equipment, special dyes or reagents, and complex post-image processing, limiting their accessibility. In this study, we demonstrate 3D-ExM, a specimen-based super-resolution microscopy approach modified from traditional ExM, which achieves consistent <30 nm lateral and <50 nm axial resolution. This method surpasses common optical or post-image processing-based super-resolution techniques and does not require specialized equipment, oxygen removal, or iterative expansion processes. Additionally, 3D-ExM can be integrated with widely used super-resolution microscopes, such as STED, to achieve further enhanced resolution. Featuring an affordable and user-friendly protocol, 3D-ExM makes super-resolution microscopy accessible to all researchers, facilitating nanoscale discoveries in various research fields.

The primary challenges in ExM technologies include the complex protocols and the difficulty of achieving uniform, isotropic 2D/3D expansions across all organelles. In this study, we demonstrated that 3D-ExM methods reliably achieve 3D-isotropic expansion, as validated through correlative pre- and post-3D-ExM analyses. Population measurements further confirmed that 3D-ExM consistently maintains 3D isotropic expansion of the nucleus in both 2D and 3D culture systems. Moreover, 3D-ExM effectively preserves protein architectures, chromatin, and RNA, as demonstrated by the visualization of EBV virions, HIV-1 genomic RNA, kinetochores, mitotic chromosomes, centrosomes, microtubules, and NPs. The 4× and 12×

3D-ExM methods are applicable to a broad range of research fields, including nuclear and chromosome research. Since ExM physically expands specimens through water absorption, it often requires imaging with a larger field of view (FOV), a greater number of z-stacks, and extended exposure time. These factors can increase the likelihood of physical drift during 3D imaging, although our imaging chambers significantly mitigate this issue. To further address the sample drift, we developed 3D-Aligner (Loi et al., 2024), a post-imaging computational tool that accurately corrects drift-induced distortion, enabling precise 3D reconstruction and accurate downstream quantification.

ExM technology is continuously advancing to improve image resolution. Achieving single-protein resolution with standard confocal microscopy would require an expansion greater than 12-fold, ideally in the range of 50- to 100-fold. Inspired by iterative expansion microscopy (iExM) (Chang et al., 2017) and its recent applications to chromatin (chromExM) (Pownall et al., 2023), combining 4× and 12× 3D-ExM or repeating 12× 3D-ExM protocol could potentially achieve expansions of ~50-fold and ~150-fold expansion. Collectively, 3D-ExM represents an affordable and straightforward super-resolution imaging tool, accessible to researchers and enabling nanoscale visualization and quantifications of protein and genomic architectures.

Materials and methods

Reagents

Immunostaining

- Phosphate-buffered saline (PBS): cat. no. P3813; Sigma-Aldrich
- Paraformaldehyde (PFA): cat. no. P6148; Sigma-Aldrich
- Nonidet P-40 substitute (NP40): SCBT, cat. no. sc-29102
- Bovine serum albumin (BSA): cat. no. A2153; Sigma-Aldrich.

Crosslinking

- 70% Glutaraldehyde solution (GA): cat. no. G7776; Sigma-Aldrich.

4× 3D-ExM gel

- Phosphate-buffered saline (PBS): cat. no. P3813; Sigma-Aldrich
- Sodium chloride (NaCl): cat. no. BP358-212; Fisher BioReagents
- Acrylamide: cat. no. A9099; Sigma-Aldrich
- N,N'-Methylenebisacrylamide (MBAA): cat. no. M7279; Sigma-Aldrich
- Sodium acrylate (SA): cat. no. 408220; Sigma-Aldrich
- 4× Monomer solution (4× 3D-ExM MS): 1x PBS, 2 M NaCl, 2.5% wt/vol Acrylamide, 0.15% wt/vol MBAA, 8.6% wt/vol SA
- 20 ml of 4× 3D-ExM MS = 2 ml of 10x PBS + 8 ml of 5 M NaCl + 1.25 ml of 40% wt/vol Acrylamide + 2 ml of 1.5% wt/vol MBAA + 1.8 g SA + ddH₂O
- Ammonium persulfate (APS): cat. no. A3678; Sigma-Aldrich
- 10% APS = 0.1 g APS + 900 µl of ddH₂O
- N,N,N',N'-Tetramethylethylenediamine (TEMED): cat. no. T7024; Sigma-Aldrich
- 10% TEMED = 2 µl of TEMED + 18 µl of ddH₂O

- 200 μ l of 4 $\times$ Gelling solution (4 $\times$ 3D-ExM GS) = 190 μ l of 4 $\times$ 3D-ExM MS + 2 μ l of ddH₂O + 4 μ l of 10% APS + 4 μ l of 10% TEMED

12 $\times$ 3D-ExM gel

- N,N-Dimethylacrylamide (DMAA): cat. no. 274135; Sigma-Aldrich
- Sodium acrylate (SA): cat. no. 408220; Sigma-Aldrich
- 12 $\times$ Monomer solution (12 $\times$ 3D-ExM MS): 1.335 g DMAA + 0.32 g SA + 2.85 ml of ddH₂O
- Potassium persulfate (KPS): cat. no. 379824; Sigma-Aldrich
- 0.036 g/ml KPS = 0.018 g KPS + 500 μ l of ddH₂O
- N,N,N',N'-Tetramethylethylenediamine (TEMED): cat. no. T7024; Sigma-Aldrich
- 200.8 μ l of 12 $\times$ Gelling solution (12 $\times$ 3D-ExM GS) = 180 μ l of 12 $\times$ 3D-ExM MS + 20 μ l of 0.036 g/ml KPS + 0.8 μ l of TEMED.

Protein digestion

- Triton X-100: cat. no. T9284; Sigma-Aldrich
- Sodium dodecyl sulfate (SDS): cat. no. 1667289; Roche
- TTSDS buffer: 1 $\times$ TAE, 0.5% Triton X-100, 1% SDS, ddH₂O
- Proteinase K (ProK): cat. no. EO0492; Thermo Fisher Scientific
- Digestion solution (DS): 16 U/ml ProK/TTSDS buffer.

Materials

- Glass slide: cat. no. 16005-106; Avantor
- Square mold (13 mm diameter $\times$ 0.8 mm depth, 25 mm $\times$ 25 mm OD): cat. no. 664107; Grace Bio-Labs
- Square cover glass (25 $\times$ 25 mm): cat. no. 2875-25; Corning
- Rectangle mold (19 mm $\times$ 32 mm $\times$ 0.8 mm depth, 25.5 mm $\times$ 44 mm OD): cat. no. 664103; Grace Bio-Labs
- Rectangle cover glass (24 $\times$ 60 mm, No. 1), cat. no. 10748-190; VWR.

Cell culture

Human HeLa, RPE1, T47D, MCF7, and rat kangaroo PtK2 cells were originally obtained from the American Type Culture Collection (ATCC). HeLa (DMEM High glucose, cat. no. SH30243.01; Cytiva), RPE1 (DMEM/F12, cat. no. SH30261.01; Cytiva), and T47D (RPMI, cat. no. SH30255.01; Cytiva) cells were grown as monolayer cultures on 12-mm # 1.5 circular coverslips in their corresponding growth media supplemented with 1% penicillin-streptomycin, 1% L-glutamine, and 10% fetal bovine serum under 5% CO₂ at 37°C in an incubator. PtK2 cells were cultured in EMEM media (cat. no. 12492013; Gibco) supplemented with 20% FBS and 1% penicillin-streptomycin, 1% L-glutamine, under 5% CO₂ at 37°C. MCF7 (gift from Andreas Friedl), or patient-derived cells were cultured as 3D spheroids in a 1:1 mixture of DMEM/High Glucose media and Matrigel (cat. no. 354230; Corning). Cells were passaged as 40 μ l droplets/well in a 24-well plate and nourished with 500 μ l media. For imaging, spheroids were dissociated into small clusters with trypsin, pelleted, and re-suspended in a 1:1 mixture of culture media and Matrigel. Patient tissue was collected with informed consent from all patients in accordance with Health Insurance Portability and Accountability Act (HIPAA) regulations, and all studies were

approved by the IRB at the University of Wisconsin-Madison (IRB# UW14035, approval no. 2014-1053). Eligible patients were planned for an ultrasound biopsy, meeting certain criteria determined by the Diagnostic Radiologist. All subjects provided written informed consent. For imaging, spheroids were dissociated into small clusters with trypsin, pelleted, and re-suspended in a 1:1 mixture of culture media and Matrigel. They were plated as 10 μ l droplets onto 12-mm # 1.5 circular coverslips in a 24-well plate, nourished with 500 μ l media until fixation.

Antibodies and dyes

The following primary antibodies were used in this study: mouse anti-Plk1 antibody (sc17783, 1:100; Santa Cruz Biotech), guinea pig anti-CENP-C antibody (PD030, 1:1,600; MBL), mouse anti-alpha Tubulin antibody (DM1A, T6199, 1:200; Sigma-Aldrich), mouse anti-Nup107 antibody (ab24609, 1:500; Abcam), rabbit anti-Elys (205696-T10, 1:100; SinoBiological), anti-GP350 antibody (40373, 1:100; SinoBiological), mouse monoclonal Gag antibody (183-H12-5C; 1:1,000) from Bruce Chesebro and obtained from the NIH AIDS Research and Reference Reagent Program (Bethesda, MD, USA) (Chesebro et al., 1992). The secondary antibodies used were Rat anti-mouse IgG2a-biotin (13-4210-80, 1:100; Thermo Fisher Scientific), Rat anti-mouse IgG1-biotin (13-4015-80, 1:100; Thermo Fisher Scientific), and minimal cross-react Alexa 488 conjugated antibodies against rabbit, mouse, and guinea pig, (711-545-152, 715-545-150, 706-155-148, 1:300; Jackson ImmunoResearch). To label biotinylated secondary antibodies, streptavidin conjugated with Alexa fluor 488 (cat. no. S32354, 1:750; Invitrogen) or Alexa fluor 568 (cat. no. S11226, 1:750; Invitrogen) was used. For DNA staining in 3D-ExM, DAPI (cat. no. D1306; Invitrogen) was used for Fig. 3; Fig. 7 B; and Fig. S6. Hoechst (cat. no. H3570; Invitrogen) was used for Fig. S6. Nuclear Green LCS1 (cat. no. ab138904; abcam) was used for Fig. S6. SYTOX Green (cat. no. S7020; Invitrogen) was used for Fig. S6. PI (cat. no. P1304MP; Invitrogen) was used for Fig. 6 C and Fig. S6. DRAQ5 (cat. no. 62251; Thermo Fisher Scientific) was used for Fig. 2; Fig. 4; Fig. 5; Fig. 6, A and B; Fig. 7 A; and Fig. S6. DAPI and Hoechst specifically labeled DNA in both 4 $\times$ and 12 $\times$ 3D-ExM. In contrast, LCS1 and SYTOX green exhibited significantly faster photobleaching in 3D-ExM compared with other DNA dyes. PI and DRAQ5 stained both DNA and cytosolic RNA, leading to increased cytoplasmic background signal without RNase treatment.

Fixation and staining for 3D-ExM (pre-embedding staining)

Cells were fixed with 4% PFA in PHEM (60 mM PIPES, 27.2 mM HEPES, 10 mM EGTA, and 8.2 mM MgSO₄) for 15 min at 37°C followed by three washes with PBS. For microtubule staining, cells were fixed with GA solution (0.8% GA, 1% Triton X-100, 3% PFA, PHEM buffer) and quenched by NaBH₄. Fixed cells were permeabilized with 0.5% NP40 at room temperature (RT) for 15 min. Cells were incubated with 0.1% BSA at RT for 30 min. Then, cells were incubated with primary antibody solution (antibodies are listed in Antibodies) and secondary antibody solution at 37°C in a humidified chamber. If biotin-conjugated secondary antibodies were used, cells were incubated with fluorophore-conjugated streptavidin at 37°C in a humidified

chamber. Crosslink was performed with 2% GA/PBS at RT for 12–16 h (overnight). A glass slide was prepared with a square mold on top. The stained coverslip was transferred into the mold with the cell side facing up (Fig. 1).

4× 3D-ExM (continued from fixation and staining)

100 µl of 4× 3D-ExM MS was added into the mold to infiltrate the cells at 4°C for 30 min. 4× 3D-ExM MS was replaced with 100 µl of 4× 3D-ExM GS and the cells were incubated at 37°C in a humidified chamber for 1 h to form the gel. 200 µl of DS was added to each gel with a piece of parafilm on top and the gel was incubated at 37°C for 3 h in a humidified chamber. The gel was transferred from the coverslip to a 10-cm dish filled with ddH₂O. The gel was incubated in ddH₂O for 3 h and ddH₂O was replaced every 30 min to allow for gel expansion.

12× 3D-ExM (continued from fixation and staining)

100 µl of 12× 3D-ExM MS was added to infiltrate the cells at RT for 10 min. 12× 3D-ExM MS was replaced with 130 µl of 12× 3D-ExM GS and the cells were incubated at RT in a humidified chamber for 2 h to form the gel. A piece of parafilm was placed on top of the mold to prevent air exposure. 200 µl of DS was added onto each gel with a piece of parafilm on top and the gel was incubated at 37°C for 24 h (overnight) in a humidified chamber. The gel was transferred from the coverslip to a 25 cm dish filled with ddH₂O. The gel was incubated in ddH₂O for at least 20 h (overnight) and ddH₂O was replaced every 1 h during the first 6 h to allow for gel expansion.

Generation and titration of HIV-1 Vif-/Vpr-CFP virus

Human embryonic kidney (HEK) 293T cells at 30–40% confluency were transfected with 10 µg plasmid DNA (9 µg plasmids encoding a biosafe, single-round HIV-1 Env-/Vif-/Vpr-/Nef-/CFP reporter virus (Evans et al., 2018; Ghone et al., 2024) with 1 µg plasmid encoding the G protein from vesicular stomatitis virus (VSV-G) (Fouchier et al., 1997) for virion pseudotyping) in 10-cm dishes using polyethylenimine (PEI; catalog no. 23966; Polysciences Inc.). The culture media was replaced 24 h after transfection. 48 h after transfection, virion-containing supernatant was harvested and filtered.

HIV-1 infection, fluorescence in situ hybridization (FISH), and immunofluorescence

RNA-FISH method for HIV-1 genome is described in a previous study (Becker et al., 2017). HeLa cells were plated on 12-mm circular coverslips (#1.5 thickness) in 12-well plates and allowed to grow to 30–40% confluency prior to infection. Cells were infected with 500 µl of the above HIV-1 Env-/Vif-/Vpr-/Nef-/CFP reporter virus using DEAE-Dextran (catalog no. 9064-91-9; Millipore Sigma) at a concentration of 6 mg/ml. 24 h after infection, culture media was replaced, and at 48 h after infection, cells were washed with PBS and fixed in 3.7% formaldehyde in PBS. Cells were permeabilized with 70% ethanol for at least 1 h at 4°C. Custom Integrated DNA Technologies (IDT) probes were designed against NL4-3 HIV-1 unspliced (US) RNA specific for the gag-pol open reading frame (nucleotides 386–4614) and containing a biotin modification of the 5'-ends of each probe (48

probes total: 5'-AACTGCGAATCGTTCTAGCT-3', 5'-ATGTCTCTAAAAGGCCAGGA-3', 5'-TGAAGGGATGGTTGTAGCTG-3', 5'-TCTTATCTAAGGCTTCCTTG-3', 5'-TACTACTTTTACCCATGCAT-3', 5'-ACATGGGTATTACTTCTGGG-3', 5'-CATGCACTGGATGCAATCTA-3', 5'-ATCCTATTTGTTCTGAAGG-3', 5'-GGGATAGGTGGATTATGTGT-3', 5'-TGGTAGGGCTATACATTCTT-3', 5'-CTCGGCTCTTAGAGTTTTAT-3', 5'-GGTTTCTGTCACTCAATTCT-3', 5'-CAATCTGGGTTCCGATTTTG-3', 5'-TTCAGCCAAAACCTCTTGCTT-3', 5'-AGTCTTTCTTTGGTTCCTAA-3', 5'-CAAACCTGAAGCTCTCTTCT-3', 5'-GAGTGATCTGAGGGAAGCTA-3', 5'-TCCGCAGATTTCTATGAGTA-3', 5'-GTCCTACTAATACTGTACTCT-3', 5'-CCATTGTTTAACTTTTGGGC-3', 5'-CCCAGAAATCTTGAGTTCTC-3', 5'-GCAGTATACTTCTGAAGTC-3', 5'-TGGAAATTGCTGGTGATCC-3', 5'-ATGTTTTTTGTCTGGTGTGG-3', 5'-GATGGAGTTCATAACCCATC-3', 5'-CCCTGCATAAATCTGACTTG-3', 5'-GTACTACTTCTGTTAGTGCT-3', 5'-TAGAATCTCCCTGTTTTCTG-3', 5'-TAATACACTCCATGTACCGG-3', 5'-TGCTTCTGTAATTTCTGCTAT-3', 5'-CATTTCTGCATACTTCTCTG-3', 5'-TCTTTCCCATATTACTATG-3', 5'-CATGTTTCTTTTGTATGG-3', 5'-CTAAGGGAGGGGTATTGACA-3', 5'-CGTTAGGGGGACAACTTTT-3', 5'-TGCTTGTAACCTCAGTCTTCT-3', 5'-TTACTTCTAATCCGAATCC-3', 5'-GTGCTTGAATGATTCCCAAT-3', 5'-TTGTTTCAATTTCTCCAATTC-3', 5'-ATAGTACTTTCTGATTCAT-3', 5'-GCCTTATCTATTCATCTAA-3', 5'-AAATCACTAGCCATTGCTCT-3', 5'-TGGCTACTATTTCTTTTGCT-3', 5'-CCCTTTTAGCTGACATTTAT-3', 5'-TACATGAAGTCCACCAAGA-3', 5'-TGGTGAAATTGCTGCCATTG-3', 5'-ACTTTGGGGATTGTAGGGA-3', 5'-TCTGCTGTCCCTGTAATAAA-3'). Cells were hybridized with the Gag/Gag-Pol IDT Biotin RNA FISH probe set using Stellaris FISH (Biosearch Technologies, Inc.) buffers and following the Stellaris FISH instructions available online at <http://www.biosearchtech.com/stellarisprotocols>. Immunofluorescence was carried out after hybridization of the biotin probes. Cells were washed with PHEM buffer and blocked in 0.1% BSA/PHEM solution for 30 min at 37°C. Primary antibody to Gag p24 (Chesebro et al., 1992) (1:100) and streptavidin secondary antibody (1:100) were diluted in blocking buffer and incubated for 3 h at 37°C. Cells were washed in PHEM prior to incubation in secondary antibody (Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 from catalog # A-11005, RRID AB_2534073; 1:300; Thermo Fisher Scientific) diluted in blocking buffer for 2 h at 37°C. An additional Gag secondary/tertiary antibody (Donkey anti-Goat IgG [H+L] Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 from catalog # A-11058, RRID AB_2534105; 1:300; Thermo Fisher Scientific) was diluted in blocking buffer and incubated with cells for 1 h at 37°C. Finally, cells were washed three times in PHEM buffer prior to 12x 3D-ExM procedure.

Imaging

Nikon Ti2 stand was equipped with Yokogawa SoRa CSU-W1 spinning disc confocal, a Yokogawa uniformizer, Hamamatsu Orca Flash4 cameras, and a high-power laser unit (100 mW for 405, 488, 561, 640 nm wavelength). Z-stack images were acquired at a step of 0.1–0.4 µm (mostly 0.4 µm for 3D-ExM images) by Nikon NIS element software (version 5.21). Most of post-3D-ExM were imaged with Plan Apo VC 60× water

objective (NA = 1.20) or Plan Apo 60× oil objective (NA = 1.40). For correlative imaging with pre- and post-3D-ExM, both samples were imaged with 20× water objective (NA = 0.95). Other pre-ExM samples were imaged with Plan Apo 100× oil objective (NA = 1.45) or Plan Apo 60× oil objective (NA = 1.40). A house-made gel chamber was used for imaging. For 12× 3D-ExM imaging, a rectangle cover glass (24 × 60 mm) with a rectangle mold on top was prepared. A razor blade was used to cut the gel into the size of 24 × 18 mm and the gel was put into the rectangle mold with the cell side facing down. For 4x 3D-ExM, a square cover glass (25 × 25 mm, #1.5) with a square mold on top was prepared. a razor blade to cut the gel into the size of 10 × 10 mm and the gel was put into the square mold with the cell side facing down. ddH₂O or house-made mounting media (30% of glycerol, 20 mM Tris, pH 8.0, 0.5% N-propyl gallate) was added into the mold to fill up the space. Notably, a higher concentration of glycerol significantly increased the brightness of ExM samples compared with lower concentrations or ddH₂O. However, this also introduced some degree of gel shrinkage. Concentrations below 30% glycerol failed to provide the brightness advantage, making 30% glycerol the optimal balance between brightness and minimal shrinkage in our experiments. That said, we observed slight shrinkage over time even at 30% glycerol, and so we recommend imaging rapidly after mounting with glycerol-based media. For most of our imaging, we either used ddH₂O for mounting or proceeded immediately after applying our house-made mounting media. A glass slide was placed on top of the mold and binder clips were used to clamp them together.

3D rendering of cellular structures in 3D-ExM images

To perform 3D rendering of individual nuclei or mitotic chromosomes, we used Imaris (version 9.5.1; Andor) to create a surface that exactly fits the shape of the nucleus in 3D. We used either the “Distance” or “Isoline” function to manually draw the contour/outline of the nucleus at every three z planes for the nucleus and every single z plane for the chromosome, and the whole surface of the cellular structure was then created automatically. The volume and the surface area of the 3D-rendered nucleus were obtained from the “Statistics” section using the Imaris software.

Determination of the expansion factor of nucleus

The lengths of the major and minor axes, as well as the nuclear area, were quantified in both pre- and post-3D-ExM images using Nikon NIS-Elements software. The nuclear volume and surface area were determined through 3D rendering with Imaris software, as described in the previous section. The axial length of the nucleus was calculated by dividing the measured volume by the average lengths of the major and minor axes.

Microtubule thickness measurement

The thickness of microtubules was determined using the full width at half maximum (FWHM) method. In Nikon Element software, a perpendicular line was drawn across a microtubule filament, and the intensity profile along this line was acquired. The FWHM of the microtubule filaments was then calculated using the software’s FWHM function.

Statistics

The data are represented as the mean ± standard deviation (s.d.). Welch’s *t*-test (two-tailed) was used to compare the means between the two populations. *P* < 0.05 was considered statistically significant. All quantifications were performed with at least two biological replicates. Sample numbers and numbers of replicates are stated in each figure legend.

Online supplemental material

Fig. S1 presents the optimization steps for crosslinking and digestion in 3D-ExM methods. **Fig. S2** provides additional 12× 3D-ExM results in organoids. **Fig. S3** displays pre- and post-3D-ExM staining of microtubules and NPs. **Fig. S4** illustrates pre- and post-12× 3D-ExM imaging of EBV and HIV-1. **Fig. S5** includes further representative images of 3D-ExM applied to centrosomes, kinetochores, and chromosomes. **Fig. S6** compares the compatibility of various DNA dyes with 3D-ExM methods. **Video 1** shows a 3D rendering of an interphase RPE1 nucleus using 12× 3D-ExM. **Videos 2, 3, and 4** depict 12× 3D-ExM images of mitotic PtK2 cells with a normal karyotype (**Video 2**), with an additional chromosome (**Video 3**), and with a chromosome loss (**Video 4**).

Data availability

Comprehensive details of the methods developed in this study can be found in the Materials and methods section. Other data and original images used in this study are available from the corresponding author (A. Suzuki) upon reasonable request.

Acknowledgments

We would like to thank Drs. Nathan Claxton, Hiroshi Nishida, Yoshitaka Sekizawa, the University of Wisconsin Optical Imaging Core, Yokogawa Electrical Corporation, Nikon Japan, and Nikon USA for critical equipment and technical support. We also would like to thank Drs. William Sugden, Beth Weaver, Robert Lera, Emily Kaufman, Yu-Lin Chen, Chieh-Chang Lin, Evelyn Wang, and Rebeca Garcia-Varela for the critical suggestions and experimental support.

Part of this work was supported by Wisconsin Partnership Program, the University of Wisconsin-Madison Office of the Vice Chancellor for Research with funding from the Wisconsin Alumni Research Foundation, start-up funding from University of Wisconsin-Madison SMPH, UW Carbone Cancer Center, and McArdle Laboratory for Cancer Research, and National Institutes of Health grant R35GM147525 and CRNA grant U54AI170660 (to A. Suzuki), R01GM131068 and R01CA234904 (to M.E. Burkard), U54AI170660, R01AI110221, and P01CA022443 (to N. Sherer), and R01GM148729 and UND COBRE pilot genomics awards (M. Takaku).

Author contributions: R.X. Norman: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - review & editing, Y.-C. Chen: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - original draft, Writing - review & editing, E.E. Recchia: Investigation, Methodology, Validation, Writing - review & editing, J. Loi: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing, Q.

Rosemarie: Investigation, Methodology, Writing - review & editing, S.L. Lesko: Investigation, Methodology, Writing - review & editing, S. Patel: Investigation, Writing - review & editing, N. Sherer: Funding acquisition, Methodology, Resources, Supervision, Validation, Writing - review & editing, M. Takaku: Conceptualization, Writing - review & editing, M.E. Burkard: Conceptualization, Funding acquisition, Resources, Supervision, Visualization, Writing - review & editing, A. Suzuki: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing - original draft.

Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. R.X. Norman reported a patent to Kits and methods for super-resolution microscopy licensed "WARF." E.E. Recchia reported a patent to US Provisional Patent application US-2022-0074829 titled "Optimized Economical and Modulatable Isotropic Expansion Microscopy" pending. M.E. Burkard reported a patent to KITS AND METHODS FOR SUPER-RESOLUTION MICROSCOPY pending "na." A. Suzuki reported a patent to US-2022-0074829 pending. No other disclosures were reported.

Submitted: 16 July 2024

Revised: 1 October 2024

Accepted: 6 November 2024

References

Amos, L.A., and T.S. Baker. 1979. The three-dimensional structure of tubulin protofilaments. *Nature*. 279:607–612. <https://doi.org/10.1038/279607a0>

Becker, J.T., N.M. Sherer, and F. Kirchhoff. 2017. Subcellular localization of HIV-1 gag-pol mRNAs regulates sites of virion assembly. *J. Virol.* 91: e02315–16. <https://doi.org/10.1128/JVI.02315-16>

Evans, E.L. III, J.T. Becker, S.L. Fricke, K. Patel, N.M. Sherer, and F. Kirchhoff. 2018. HIV-1 vif's capacity to manipulate the cell cycle is species specific. *J. Virol.* 92:e02102–17. <https://doi.org/10.1128/JVI.02102-17>

Beese, L., G. Stubbs, and C. Cohen. 1987. Microtubule structure at 18 Å resolution. *J. Mol. Biol.* 194:257–264. [https://doi.org/10.1016/0022-2836\(87\)90373-1](https://doi.org/10.1016/0022-2836(87)90373-1)

Bettencourt-Dias, M., and D.M. Glover. 2007. Centrosome biogenesis and function: Centrosomes brings new understanding. *Nat. Rev. Mol. Cell Biol.* 8:451–463. <https://doi.org/10.1038/nrm2180>

Booth, D.G., A.J. Beckett, O. Molina, I. Samejima, H. Masumoto, N. Kouprina, V. Larionov, I.A. Prior, and W.C. Earnshaw. 2016. 3D-CLEM reveals that a major portion of mitotic chromosomes is not chromatin. *Mol. Cell.* 64: 790–802. <https://doi.org/10.1016/j.molcel.2016.10.009>

Bowler, M., D. Kong, S. Sun, R. Nanjundappa, L. Evans, V. Farmer, A. Holland, M.R. Mahjoub, H. Sui, and J. Lencarek. 2019. High-resolution characterization of centriole distal appendage morphology and dynamics by correlative STORM and electron microscopy. *Nat. Commun.* 10:993. <https://doi.org/10.1038/s41467-018-08216-4>

Büttner, M., C.B. Lagerholm, D. Waithe, S. Galiani, W. Schliebs, R. Erdmann, C. Eggeling, and K. Reglinski. 2020. Challenges of using expansion microscopy for super-resolved imaging of cellular organelles. *Chem-BioChem.* 22:686–693. <https://doi.org/10.1002/cbic.202000571>

Capelson, M., and M.W. Hetzer. 2009. The role of nuclear pores in gene regulation, development and disease. *EMBO Rep.* 10:697–705. <https://doi.org/10.1038/embor.2009.147>

Chang, J.B., F. Chen, Y.G. Yoon, E.E. Jung, H. Babcock, J.S. Kang, S. Asano, H.J. Suk, N. Pak, P.W. Tillberg, et al. 2017. Iterative expansion microscopy. *Nat. Methods.* 14:593–599. <https://doi.org/10.1038/nmeth.4261>

Chang, T.B., J.C. Hsu, and T.T. Yang. 2023. Single-molecule localization microscopy reveals the ultrastructural constitution of distal appendages in expanded mammalian centrioles. *Nat. Commun.* 14:1688. <https://doi.org/10.1038/s41467-023-37342-x>

Chen, F., P.W. Tillberg, and E.S. Boyden. 2015. Optical imaging. Expansion microscopy. *Science*. 347:543–548. <https://doi.org/10.1126/science.1260088>

Chen, J., S.A. Rahman, O.A. Nikolaitchik, D. Grunwald, L. Sardo, R.C. Burdick, S. Plisov, E. Liang, S. Tai, V.K. Pathak, and W.-S. Hu. 2016. HIV-1 RNA genome dimerizes on the plasma membrane in the presence of Gag protein. *Proc. Natl. Acad. Sci. USA*. 113:E201–E208. <https://doi.org/10.1073/pnas.1518572113>

Chen, B., M. Yusuf, T. Hashimoto, A.K. Estandarte, G. Thompson, and I. Robinson. 2017. Three-dimensional positioning and structure of chromosomes in a human prophase nucleus. *Sci. Adv.* 3:e1602231. <https://doi.org/10.1126/sciadv.1602231>

Chesebro, B., K. Wehrly, J. Nishio, and S. Perryman. 1992. Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell-tropic isolates: Definition of critical amino acids involved in cell tropism. *J. Virol.* 66:6547–6554. <https://doi.org/10.1128/jvi.66.11.6547-6554.1992>

Chiu, Y.F., A.U. Sugden, and B. Sugden. 2013. Epstein-Barr viral productive amplification reprograms nuclear architecture, DNA replication, and histone deposition. *Cell Host Microbe*. 14:607–618. <https://doi.org/10.1016/j.chom.2013.11.009>

Cooke, C.A., R.L. Bernat, and W.C. Earnshaw. 1990. CENP-B: A major human centromere protein located beneath the kinetochore. *J. Cell Biol.* 110: 1475–1488. <https://doi.org/10.1083/jcb.110.5.1475>

DeLuca, J.G., Y. Dong, P. Hergert, J. Strauss, J.M. Hickey, E.D. Salmon, and B.F. McEwen. 2005. Hec1 and nuf2 are core components of the kinetochore outer plate essential for organizing microtubule attachment sites. *Mol. Biol. Cell.* 16:519–531. <https://doi.org/10.1091/mbc.e04-09-0852>

Desai, A., and T.J. Mitchison. 1997. Microtubule polymerization dynamics. *Annu. Rev. Cell Dev. Biol.* 13:83–117. <https://doi.org/10.1146/annurev.cellbio.13.1.83>

Dong, Y., K.J. Vanden Beldt, X. Meng, A. Khodjakov, and B.F. McEwen. 2007. The outer plate in vertebrate kinetochores is a flexible network with multiple microtubule interactions. *Nat. Cell Biol.* 9:516–522. <https://doi.org/10.1038/ncb1576>

Finardi, A., L.F. Massari, and R. Visintin. 2020. Anaphase bridges: Not all natural fibers are healthy. *Genes*. 11:902. <https://doi.org/10.3390/genes11080902>

Fouchier, R.A., B.E. Meyer, J.H. Simon, U. Fischer, and M.H. Malim. 1997. HIV-1 infection of non-dividing cells: Evidence that the amino-terminal basic region of the viral matrix protein is important for gag processing but not for post-entry nuclear import. *EMBO J.* 16:4531–4539. <https://doi.org/10.1093/emboj/16.15.4531>

Frappier, L. 2021. Epstein-Barr virus: Current questions and challenges. *Tumour Virus Res.* 12:200218. <https://doi.org/10.1016/j.tivr.2021.200218>

Germi, R., G. Effantin, L. Grossi, R.W.H. Ruigrok, P. Morand, and G. Schoehn. 2012. Three-dimensional structure of the Epstein-Barr virus capsid. *J. Gen. Virol.* 93:1769–1773. <https://doi.org/10.1099/vir.0.043265-0>

Ghone, D.A., E.L. Evans III, M. Bandini, K.G. Stephenson, N.M. Sherer, and A. Suzuki. 2024. HIV-1 Vif disrupts phosphatase feedback regulation at the kinetochore, leading to a pronounced pseudo-metaphase arrest. *Elife*. 13:RP101136.

Glaser, R., and F. Rapp. 1972. Rescue of Epstein-Barr virus from somatic cell hybrids of Burkitt lymphoblastoid cells. *J. Virol.* 10:288–296. <https://doi.org/10.1128/jvi.10.2.288-296.1972>

Hell, S.W., and J. Wichmann. 1994. Breaking the diffraction resolution limit by stimulated emission: Stimulated-emission-depletion fluorescence microscopy. *Opt. Lett.* 19:780–782. <https://doi.org/10.1364/OL.19.000780>

Huang, G., X. Zhan, C. Zeng, X. Zhu, K. Liang, Y. Zhao, P. Wang, Q. Wang, Q. Zhou, Q. Tao, et al. 2022. Cryo-EM structure of the nuclear ring from *Xenopus laevis* nuclear pore complex. *Cell Res.* 32:349–358. <https://doi.org/10.1038/s41422-021-00610-w>

Keane, S.C., and M.F. Summers. 2016. NMR studies of the structure and function of the HIV-1 5'-leader. *Viruses*. 8:338. <https://doi.org/10.3390/v8120338>

Kieff, E., T. Dambaugh, M. Heller, W. King, A. Cheung, V. van Santen, M. Hummel, C. Beisel, S. Fennwald, K. Hennessy, and T. Heineman. 1982. The biology and chemistry of Epstein-Barr virus. *J. Infect. Dis.* 146: 506–517. <https://doi.org/10.1093/infdis/146.4.506>

Klaassen, S.J., M.A. Truong, R.H. van Jaarsveld, I. Koprivec, V. Štimac, S.G. de Vries, P. Risteski, S. Kodba, K. Vukušić, K.L. de Luca, et al. 2022. Nuclear chromosome locations dictate segregation error frequencies. *Nature*. 607:604–609. <https://doi.org/10.1038/s41586-022-04938-0>

Knoener, R., E. Evans III, J.T. Becker, M. Scalf, B. Benner, N.M. Sherer, and L.M. Smith. 2021. Identification of host proteins differentially

- associated with HIV-1 RNA splice variants. *Elife*. 10:e62470. <https://doi.org/10.7554/eLife.62470>
- Leibowitz, M.L., C.Z. Zhang, and D. Pellman. 2015. Chromothripsis: A new mechanism for rapid karyotype evolution. *Annu. Rev. Genet.* 49:183–211. <https://doi.org/10.1146/annurev-genet-120213-092228>
- Liu, W.J., K.C. Toussaint Jr., C. Okoro, D.Z. Zhu, Y.H. Chen, C.F. Kuang, and X. Liu. 2018. Breaking the axial diffraction limit: A guide to axial super-resolution fluorescence microscopy. *Laser Photon. Rev.* <https://doi.org/10.1002/lpor.201700333>
- Loi, J., X. Qu, and A. Suzuki. 2023. Semi-automated 3D fluorescence speckle analyzer (3D-Speckler) for microscope calibration and nanoscale measurement. *J. Cell Biol.* 222:e202202078. <https://doi.org/10.1083/jcb.202202078>
- Loi, J., D. Ghone, X. Qu, and A. Suzuki. 2024. 3D-Aligner: Advanced computational tool for correcting image distortion in expansion microscopy. *Commun. Biol.* 7:1325. <https://doi.org/10.1038/s42003-024-07035-2>
- Lorenz, P.R., and J.W. Ainsworth. 1972. Chromosomes and DNA-replication of rat kangaroo cells (PtK 2). *Chromosoma*. 38:431–440. <https://doi.org/10.1007/BF00302161>
- Louten, J. 2016. Virus structure and classification. In *Essential Human Virology*. Academic Press, Cambridge, MA, USA, 19–29. <https://doi.org/10.1016/B978-0-12-800947-5.00002-8>
- Magidson, V., J. He, J.G. Ault, C.B. O'Connell, N. Yang, I. Tikhonenko, B.F. McEwen, H. Sui, and A. Khodjakov. 2016. Unattached kinetochores rather than intrakinetochore tension arrest mitosis in taxol-treated cells. *J. Cell Biol.* 212:307–319. <https://doi.org/10.1083/jcb.201412139>
- Marshall, O.J., A.T. Marshall, and K.H. Choo. 2008. Three-dimensional localization of CENP-A suggests a complex higher order structure of centromeric chromatin. *J. Cell Biol.* 183:1193–1202. <https://doi.org/10.1083/jcb.200804078>
- Musacchio, A., and A. Desai. 2017. A molecular view of kinetochore assembly and function. *Biology*. 6:5. <https://doi.org/10.3390/biology6010005>
- M'Saad, O., and J. Bewersdorf. 2020. Light microscopy of proteins in their ultrastructural context. *Nat. Commun.* 11:3850. <https://doi.org/10.1038/s41467-020-17523-8>
- Nanbo, A., T. Noda, and Y. Ohba. 2018. Epstein-barr virus acquires its final envelope on intracellular compartments with golgi markers. *Front. Microbiol.* 9:454. <https://doi.org/10.3389/fmicb.2018.00454>
- Neil, M.A.A., R. Juskaitis, and T. Wilson. 1997. Method of obtaining optical sectioning by using structured light in a conventional microscope. *Opt. Lett.* 22:1905–1907. <https://doi.org/10.1364/OL.22.001905>
- Odumade, O.A., K.A. Hogquist, and H.H. Balfour Jr. 2011. Progress and problems in understanding and managing primary Epstein-Barr virus infections. *Clin. Microbiol. Rev.* 24:193–209. <https://doi.org/10.1128/CMR.00044-10>
- Ogembo, J.G., M.R. Murawski, L.W. McGinnes, A. Parcharidou, R. Sutiwise-sak, T. Tison, J. Avendano, D. Agnani, R.W. Finberg, T.G. Morrison, and J.D. Fingerroth. 2015. A chimeric EBV gp350/220-based VLP replicates the virion B-cell attachment mechanism and elicits long-lasting neutralizing antibodies in mice. *J. Transl. Med.* 13:50. <https://doi.org/10.1186/s12967-015-0415-2>
- Ori, A., N. Banterle, M. Iskar, A. Andrés-Pons, C. Escher, H. Khanh Bui, L. Sparks, V. Solis-Mezarino, O. Rinner, P. Bork, et al. 2013. Cell type-specific nuclear pores: A case in point for context-dependent stoichiometry of molecular machines. *Mol. Syst. Biol.* 9:648. <https://doi.org/10.1038/msb.2013.4>
- Pernal, S.P., A. Liyanaarachchi, D.L. Gatti, B. Formosa, R. Pulvender, E.R. Kuhn, R. Ramos, A.R. Naik, K. George, S. Arslanturk, et al. 2020. Nanoscale imaging using differential expansion microscopy. *Histochem. Cell Biol.* 153:469–480. <https://doi.org/10.1007/s00418-020-01869-7>
- Pownall, M.E., L. Miao, C.E. Vejnar, O. M'Saad, A. Sherrard, M.A. Frederick, M.D.J. Benitez, C.W. Boswell, K.S. Zaret, J. Bewersdorf, and A.J. Gir-aldez. 2023. Chromatin expansion microscopy reveals nanoscale organization of transcription and chromatin. *Science*. 381:92–100. <https://doi.org/10.1126/science.ade5308>
- Remya, R.S., S. Hariharan, V. Keerthi, and C. Gopakumar. 2019. Pre-processing G-banded metaphase: Towards the design of automated karyotyping. *SN Appl. Sci.* 1:1710. <https://doi.org/10.1007/s42452-019-1754-z>
- Rust, M.J., M. Bates, and X. Zhuang. 2006. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods*. 3: 793–795. <https://doi.org/10.1038/nmeth929>
- Sacristan, C., K. Samejima, L.A. Ruiz, M. Deb, M.L.A. Lambers, A. Buckle, C.A. Brackley, D. Robertson, T. Hori, S. Webb, et al. 2024. Vertebrate centromeres in mitosis are functionally bipartite structures stabilized by cohesin. *Cell*. 187:3006–3023.e26. <https://doi.org/10.1016/j.cell.2024.04.014>
- Sahabandu, N., D. Kong, V. Magidson, R. Nanjundappa, C. Sullenberger, M.R. Mahjoub, and J. Loncarek. 2019. Expansion microscopy for the analysis of centrosomes and cilia. *J. Microsc.* 276:145–159. <https://doi.org/10.1111/jmi.12841>
- Sansregret, L., B. Vanhaesebroeck, and C. Swanton. 2018. Determinants and clinical implications of chromosomal instability in cancer. *Nat. Rev. Clin. Oncol.* 15:139–150. <https://doi.org/10.1038/nrclinonc.2017.198>
- Strambio-De-Castilla, C., M. Niepel, and M.P. Rout. 2010. The nuclear pore complex: Bridging nuclear transport and gene regulation. *Nat. Rev. Mol. Cell Biol.* 11:490–501. <https://doi.org/10.1038/nrm2928>
- Sugden, B. 2014. Epstein-Barr virus: The path from association to causality for a ubiquitous human pathogen. *PLoS Biol.* 12:e1001939. <https://doi.org/10.1371/journal.pbio.1001939>
- Suzuki, A., T. Hori, T. Nishino, J. Usukura, A. Miyagi, K. Morikawa, and T. Fukagawa. 2011. Spindle microtubules generate tension-dependent changes in the distribution of inner kinetochore proteins. *J. Cell Biol.* 193:125–140. <https://doi.org/10.1083/jcb.201012050>
- Suzuki, A., S.K. Long, and E.D. Salmon. 2018. An optimized method for 3D fluorescence co-localization applied to human kinetochore protein architecture. *Elife*. 7:e32418. <https://doi.org/10.7554/eLife.32418>
- Tillberg, P.W., F. Chen, K.D. Piatkevich, Y. Zhao, C.C. Yu, B.P. English, L. Gao, A. Martorell, H.J. Suk, F. Yoshida, et al. 2016. Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies. *Nat. Biotechnol.* 34:987–992. <https://doi.org/10.1038/nbt.3625>
- Truckenbrodt, S., M. Maidorn, D. Crzan, H. Wildhagen, S. Kabatas, and S.O. Rizzoli. 2018. X10 expansion microscopy enables 25-nm resolution on conventional microscopes. *EMBO Rep.* 19:e45836. <https://doi.org/10.15252/embr.201845836>
- Truckenbrodt, S., C. Sommer, S.O. Rizzoli, and J.G. Danzl. 2019. A practical guide to optimization in X10 expansion microscopy. *Nat. Protoc.* 14: 832–863. <https://doi.org/10.1038/s41596-018-0117-3>
- Valli, J., A. Garcia-Burgos, L.M. Rooney, B. Vale de Melo E Oliveira, R.R. Duncan, and C. Rickman. 2021. Seeing beyond the limit: A guide to choosing the right super-resolution microscopy technique. *J. Biol. Chem.* 297:100791. <https://doi.org/10.1016/j.jbc.2021.100791>
- von Appen, A., J. Kosinski, L. Sparks, A. Ori, A.L. DiGiulio, B. Vollmer, M.-T. Mackmull, N. Banterle, L. Parca, P. Kastiris, et al. 2015. In situ structural analysis of the human nuclear pore complex. *Nature*. 526:140–143. <https://doi.org/10.1038/nature15381>
- Weber, K., P.C. Rathke, and M. Osborn. 1978. Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells by electron microscopy and by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. USA*. 75: 1820–1824. <https://doi.org/10.1073/pnas.75.4.1820>
- Willingham, M.C. 1983. An alternative fixation-processing method for preembedding ultrastructural immunocytochemistry of cytoplasmic antigens: The GBS (glutaraldehyde-borohydride-saponin) procedure. *J. Histochem. Cytochem.* 31:791–798. <https://doi.org/10.1177/31.6.6404984>
- Yin, H., J. Qu, Q. Peng, and R. Gan. 2018. Molecular mechanisms of EBV-driven cell cycle progression and oncogenesis. *Med. Microbiol. Immunol.* 208:573–583. <https://doi.org/10.1007/s00430-018-0570-1>

Supplemental material

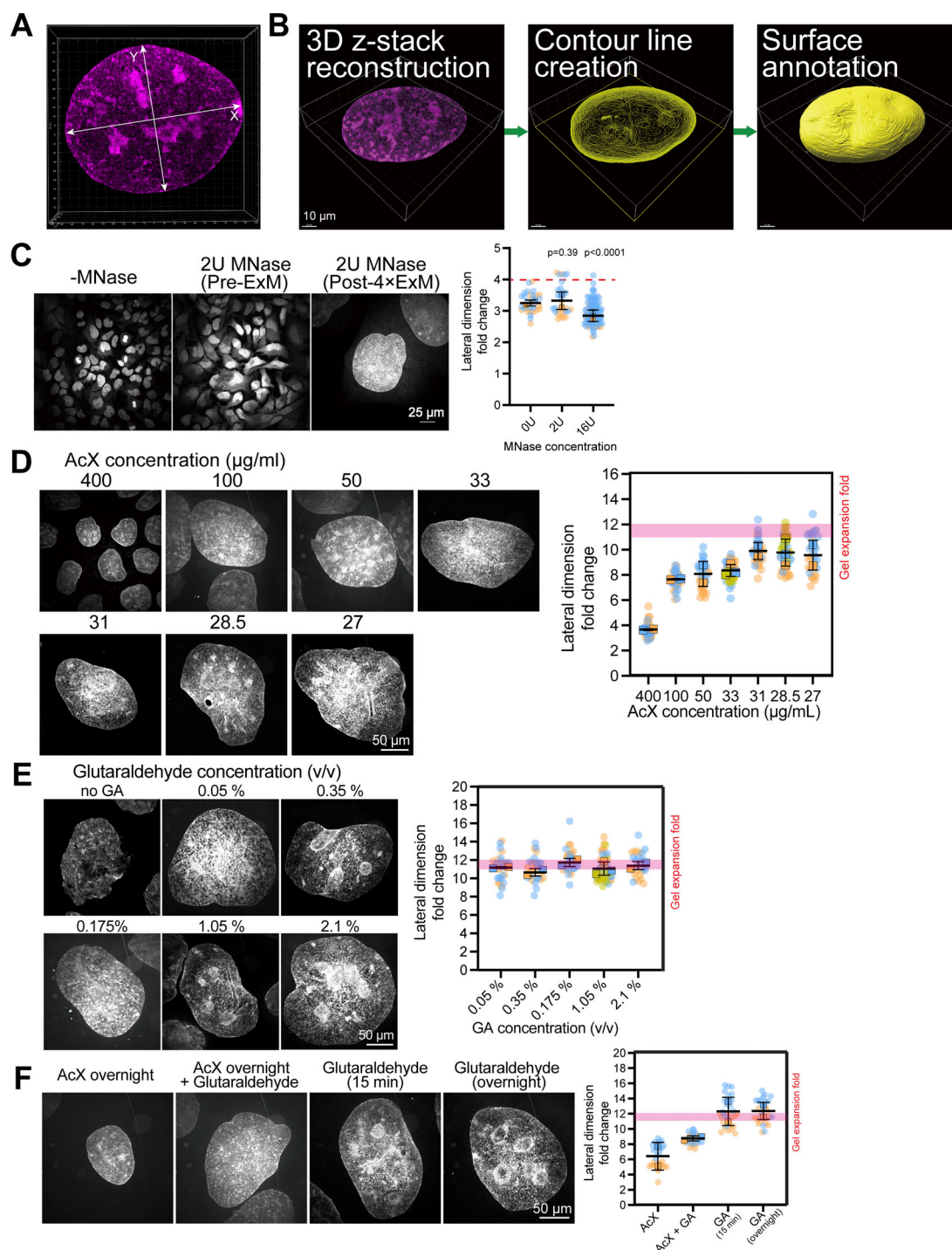


Figure S1. **Optimization of the crosslinking step in the 12x 3D-ExM protocol.** (A) An example 3D image of how the lateral and axial expansion factors were determined. The longest length along the major axis and the minor axis of an oval-shaped nucleus were used to determine the lateral expansion factor of the nucleus. (B) An example image of how a surface annotation was created to fit the shape and volume of a nucleus. (C) Left: representative images of HeLa cells treated with or without MNase before and after 4x ExM. Right: quantification of the size of the nucleus of untreated cells or cells treated with 2 U or 16 U MNase using the traditional 4x ExM protocol. (D) Optimization of the concentration of AcX as a crosslinking reagent. Left: representative images of HeLa nuclei when different concentrations of AcX were used for crosslinking in the 12x ExM protocol. Right: quantification of the size of the nucleus of HeLa cells shown on the left. $n = 46, 30, 34, 46, 27, 44$, and 29 cells (from left to right) pooled from two to three color-coded biological replicates. (E) Optimization of the concentration of GA as a crosslinking reagent. Left: representative images of HeLa nucleus when different concentrations of GA were used for crosslinking in the 12x ExM protocol. Right: quantification of the size of nucleus shown on the left. $n = 30, 30, 30, 45, 30$ cells (from left to right) pooled from two to three color-coded biological replicates. (F) Optimization of the crosslinking method for the 12x ExM protocol. Left: representative images of HeLa nucleus when cells were incubated with AcX overnight, AcX + GA overnight, GA for 15 min, or GA overnight. Right: quantification of the size of the nucleus shown on the left. $n = 30, 30, 31, 31$ cells (from left to right) pooled from two color-coded biological replicates. Each dot shown in the quantification plots indicates a single nucleus. DNA was stained with DAPI.

A Patient-derived organoid

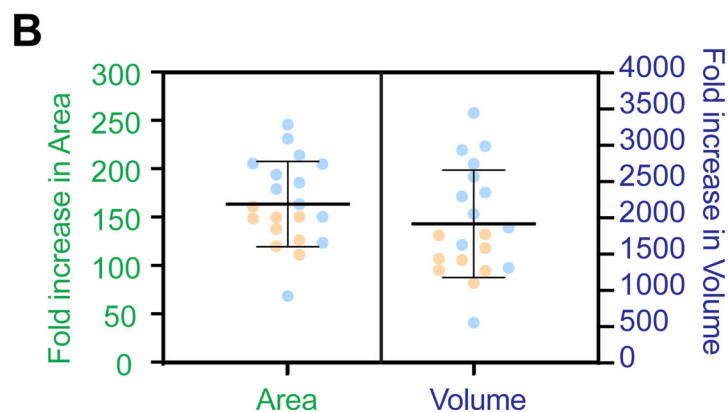
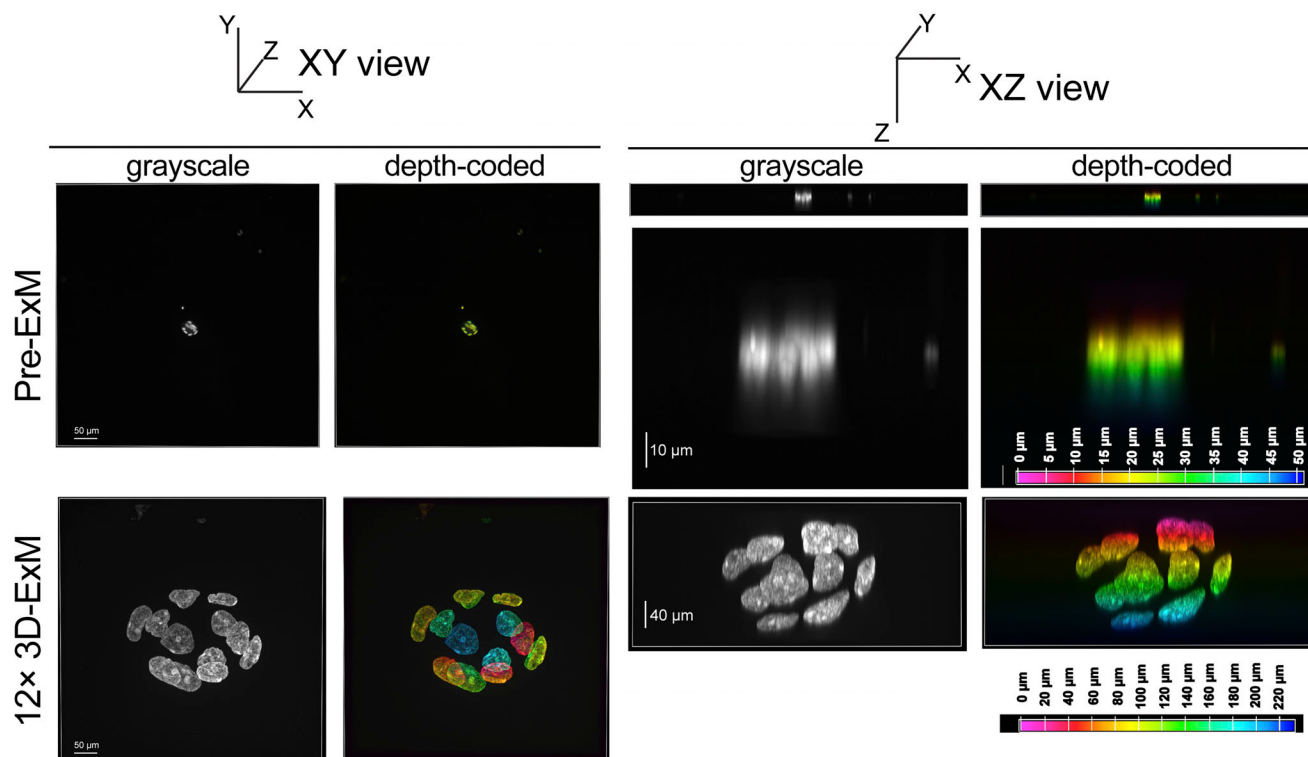


Figure S2. **12x 3D-ExM enables the expansion of patient-derived organoids by ~12-fold.** (A) Representative images of patient-derived organoids before and after 12x 3D-ExM. The XY view and XZ view of the z-stack images are shown. The depth of the objects in the z-stack image (distance from the coverslip surface) are labeled with different colors. DNA was stained with DRAQ5. (B) Quantification of the fold change of area and volume of the MCF10A organoids before and after 12x 3D-ExM. $n = 20$ cells pooled from two color-coded biological replicates. Each dot shown in the quantification plot indicates a single nucleus.

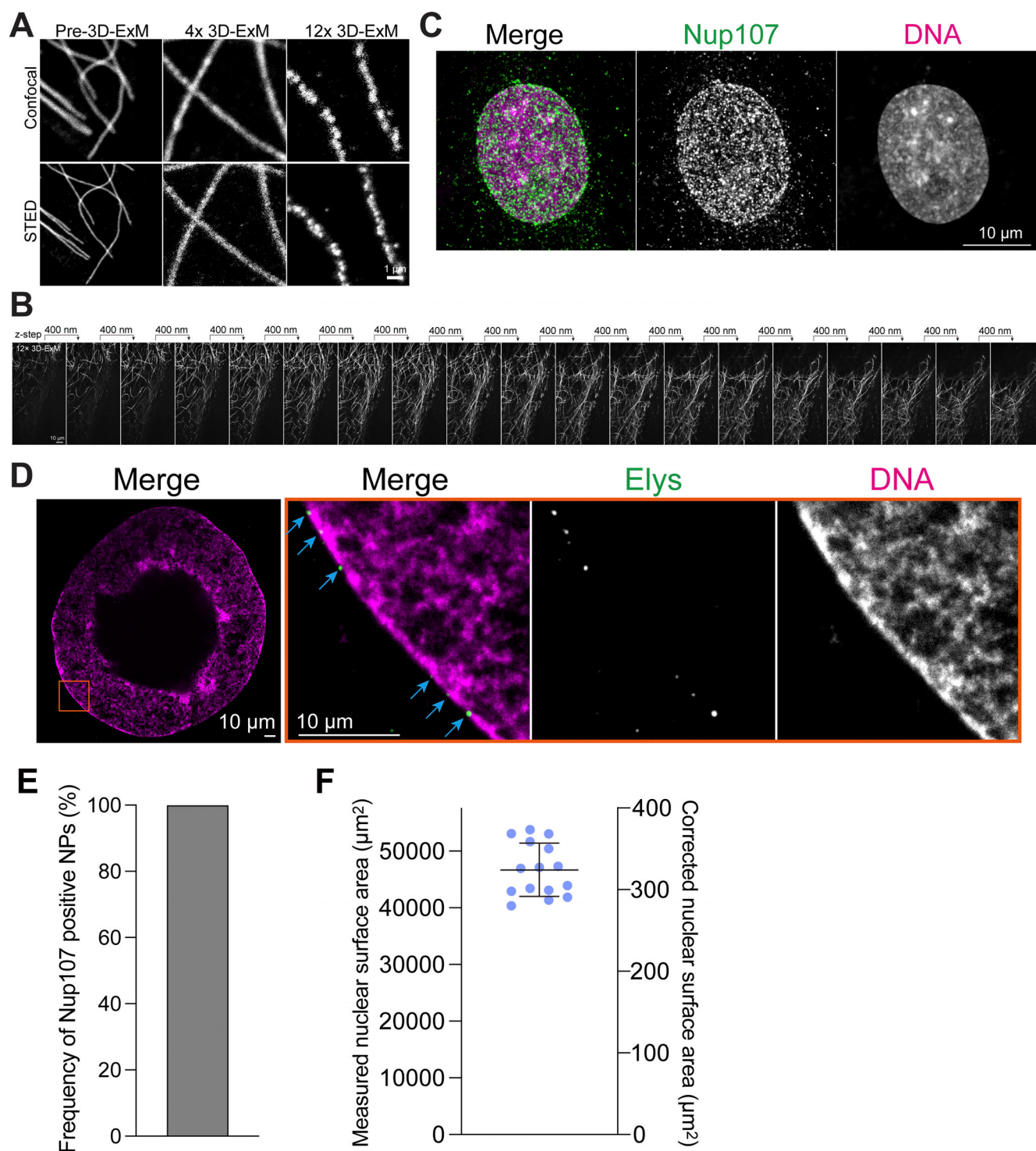


Figure S3. **Validation of the resolution limit of 3D-ExM using microtubules and NPCs.** (A) Representative images of microtubules with or without using different ExM methods (pre-ExM, 4x, and 12x 3D-ExM) and microscopes (conventional confocal and STED). (B) Consecutive serial sections (400 nm for each step) of antibody-stained microtubules in PtK2 cells after 12x 3D-ExM. (C) Representative images of RPE1 cells stained with anti-Nup107 antibody and DNA dye before expansion. (D) Representative 12x 3D-ExM images of RPE1 cells stained with anti-Elys antibody. Blue arrows indicate NPs at the edge of the nucleus. The three images within the orange bounding box are zoomed-in images of the indicated area in the left image. (E) Quantification of the frequency of NPs possessing Nup107 dots. $n = 751$ NPs pooled from six cells from two biological replicates. (F) Quantification of the measured and corrected surface area of RPE1 nuclei in 12x 3D-ExM images. $n = 15$ cells pooled from three biological replicates. DNA was stained with DAPI (pre-ExM) or DRAQ5 (post-ExM).

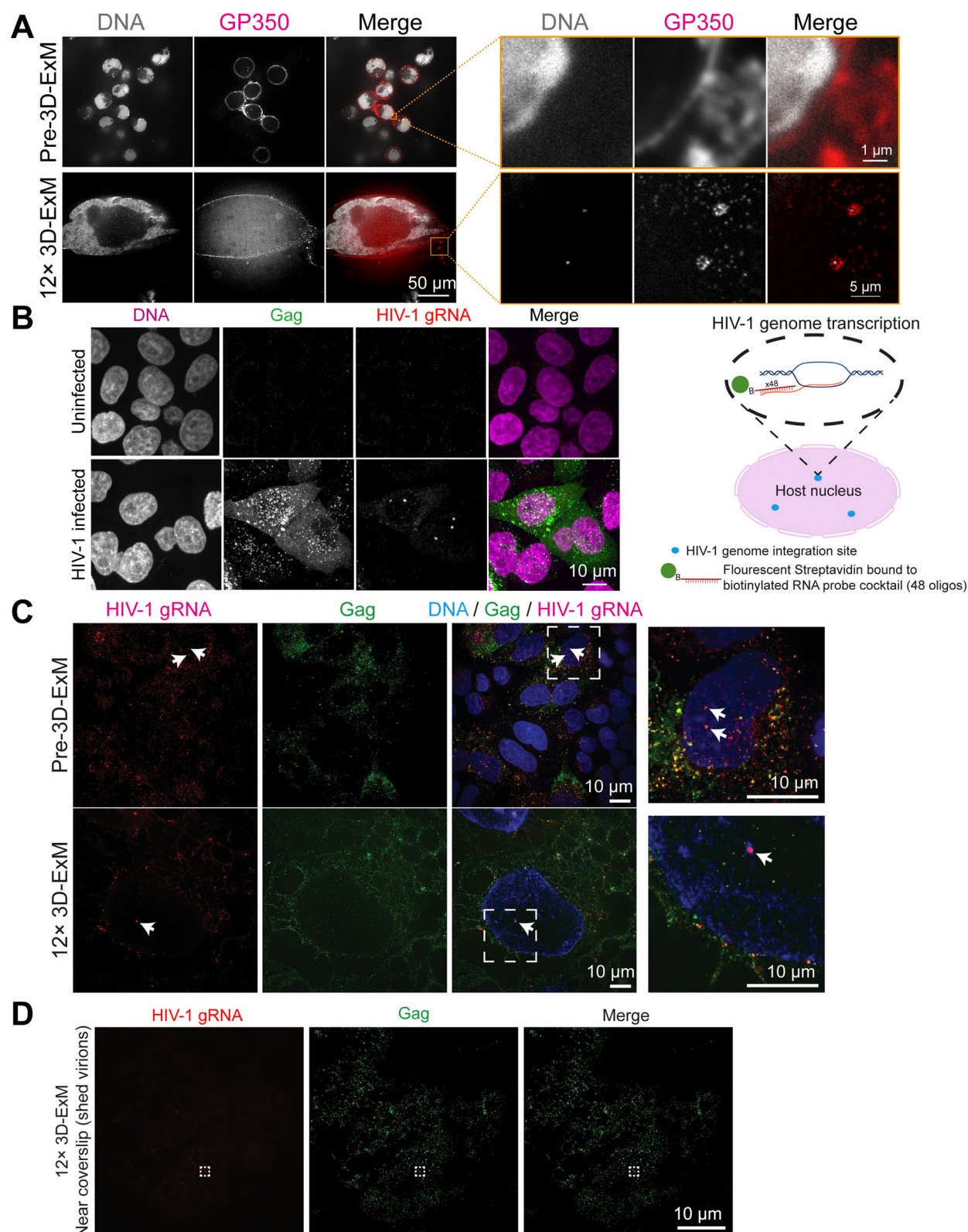


Figure S4. **Application of 3D-ExM in visualizing virus architecture.** (A) Representative images of iD98HR1 cells that have been induced to enter EBV's lytic phase and stained for the EBV envelope protein GP350 (red) and DNA (white) before and after 12x 3D-ExM. Images on the right are the zoom-in images shown in the orange box on the left images. (B) Left: representative images of uninfected and HIV-1 infected HeLa cells stained with DNA (magenta), HIV Gag (green) and HIV gRNA (red). Right: schematic diagram of how HIV genome integration sites were visualized using HIV genome sequence-specific biotinylated RNA probes. (C) Representative 12x 3D-ExM images of HIV gRNA and Gag proteins. HIV gRNA was labeled using specific FISH probes. Arrows show integration sites of HIV-1 genome in a nucleus. (D) Larger FOV images of Fig. 6 B. The enlarged images within the square boxes are shown in Fig. 6 B. DNA was stained with DAPI (pre-ExM) or DRAQ5 (post-ExM).

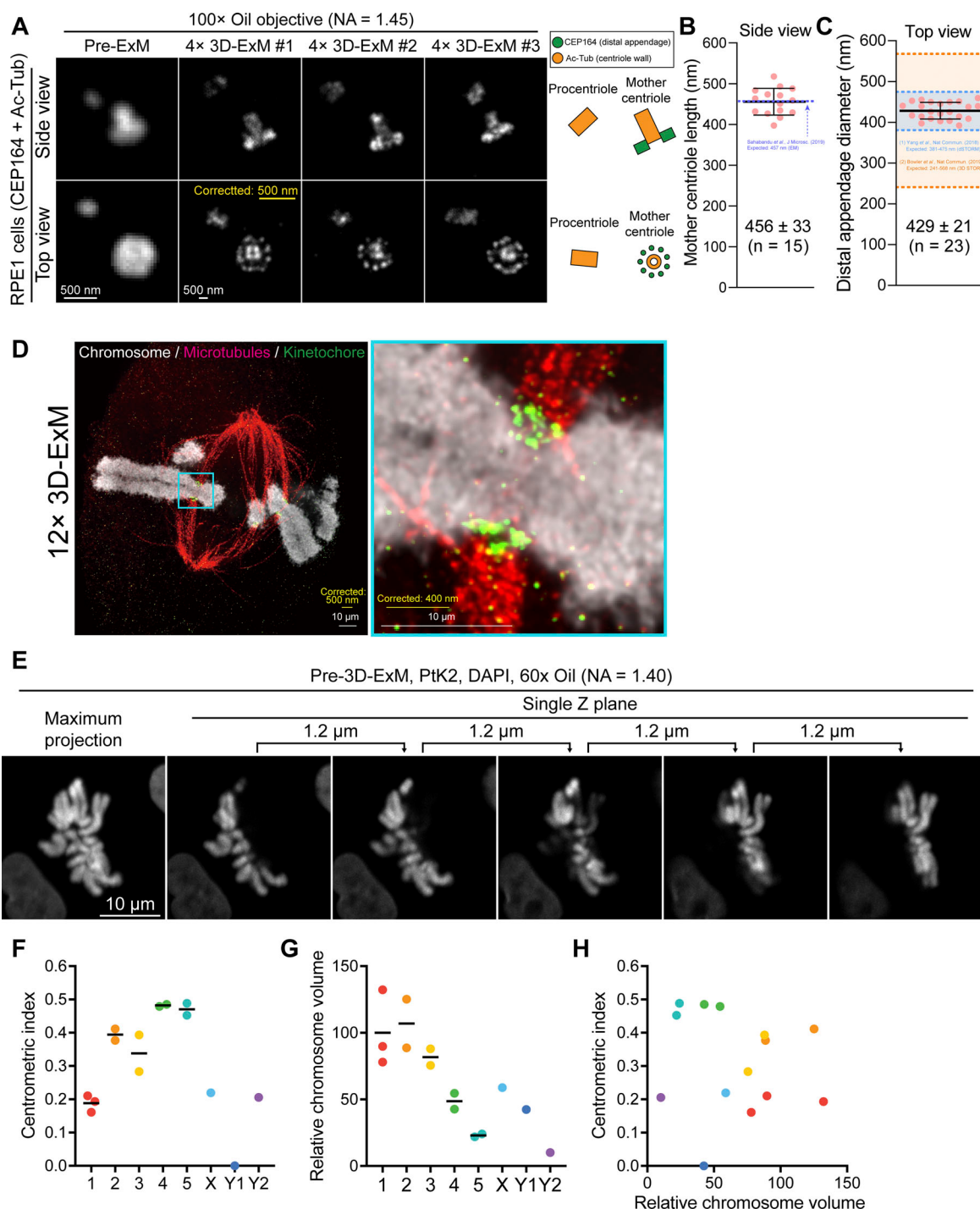


Figure S5. Application of 3D-ExM in visualizing centriole architecture, kinetochore structure, and karyotype determination. (A) Left: representative images of the centriole structure in RPE1 cells from the top view and side view perspectives before and after 4× 3D-ExM. Three 4× 3D-ExM example images of different centrioles were shown. Both Pre-3D-ExM and 4× 3D-ExM images were acquired using 100× oil objective (NA = 1.45). Right: the schematic diagram of centriole structures and the distribution of target molecules (CEP164 and acetylated-Tubulin) from the top view and side view. Note that both anti-CEP164 and anti-Ac-Tub antibodies were raised in mice, and thus the same anti-mouse secondary antibody was used for both targets in this experiment. (B) Quantification of mother centriole length and the expected length obtained from the previous study. Each dot represents a single centriole. $n = 15$ centrioles. (C) Quantification of distal appendage diameter from the top view perspective and the expected size obtained from the previous studies. Each dot represents a single centriole. $n = 23$ centrioles. (D) Left: an example 12× 3D-ExM image labeled by anti-Hec1 and anti-Tubulin antibodies in PtK2 metaphase cells. Right: the zoom-in image of the highlighted region shown in the left image. (E) Representative pre-3D-ExM images of a metaphase PtK2 cell stained with DAPI. Both maximum projection and consecutive single Z plane images (interval: 1.2 μm) are shown. Note that individual chromosomes cannot be identified with this image resolution. (F) The centromeric index of each chromosome in the normal karyotype is shown in Fig. 8 A. (G) The relative chromosome volume of each PtK2 chromosome in the normal karyotype is shown in Fig. 8 A. (H) The centromeric index vs relative chromosome volume of each PtK2 chromosome in the normal karyotype shown in Fig. 8 A.

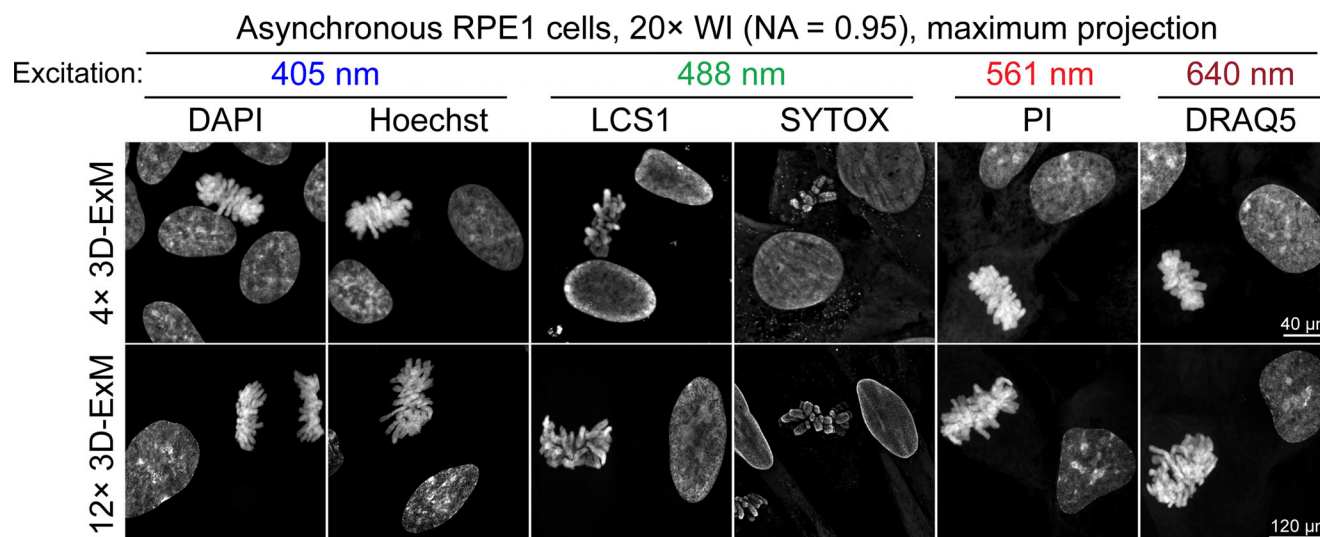


Figure S6. **Comparison of the compatibility of DNA dye with 3D-ExM methods.** Performance of different DNA dyes (DAPI, Hoechst 33342, Nuclear Green LCS1, SYTOX Green, PI, and DRAQ5) in 4× and 12× 3D-ExM using asynchronous RPE1 cells. 20× water objective (NA = 0.95) was used for this imaging.

Video 1. **An example 12× 3D-ExM 3D movie of a RPE1 interphase nucleus.** The asynchronous RPE1 interphase nucleus was stained with DNA dye (magenta) and imaged using a spinning disk confocal microscope at 400-nm intervals. The resulting movie was rendered using Imaris software. Related to Fig. 2 D.

Video 2. **An example 12× 3D-ExM 3D movie of a PtK2 metaphase cell with wildtype karyotype.** The asynchronous PtK2 metaphase cell with normal karyotype (14 chromosomes) was fluorescently labeled with kinetochores (magenta), microtubules (green), and DNA (white), and imaged using a spinning disk confocal microscope at 400-nm intervals. The resulting movie was rendered using Imaris software. Related to Fig. 8 A.

Video 3. **An example 12× 3D-ExM 3D movie of a PtK2 metaphase cell with aneuploidy (gain).** The asynchronous PtK2 metaphase cell with aneuploidy (15 chromosomes) was fluorescently labeled with kinetochores (magenta), microtubules (green), and DNA (white), and imaged using a spinning disk confocal microscope at 400 nm intervals. The resulting movie was rendered using Imaris software. Related to Fig. 8 A.

Video 4. **An example 12× 3D-ExM 3D movie of a PtK2 metaphase cell with aneuploidy (loss).** The asynchronous PtK2 metaphase cell with aneuploidy (13 chromosomes) was fluorescently labeled with kinetochores (magenta), microtubules (green), and DNA (white), and imaged using a spinning disk confocal microscope at 400 nm intervals. The resulting movie was rendered using Imaris software. Related to Fig. 8 A.