Correlative three-dimensional super-resolution and block-face electron microscopy of whole vitreously frozen cells


INTRODUCTION: Our textbook understanding of the nanoscale organization of the cell and its relationship to the thousands of proteins that drive cellular metabolism comes largely from a synthesis of biochemistry, molecular biology, and electron microscopy, and is therefore speculative in its details. Correlative super-resolution (SR) fluorescence and electron microscopy (EM) promises to elucidate these details by directly visualizing the nanoscale relationship of specific proteins in the context of the global cellular ultrastructure. However, to date such correlative imaging has involved compromises with respect to ultrastructure preservation and imaging sensitivity, resolution, and/or field of view.

RATIONALE: We developed a pipeline to (i) preserve fluorescently labeled, cultured mammalian cells in vitreous ice; (ii) image selected cells in their entirety below 10 K by multicolor three-dimensional structured illumination (3D SIM) and single-molecule localization microscopy (SMLM); (iii) image the same cells by 3D focused ion beam scanning EM (FIB-SEM) at 4- or 8-nm isotropic resolution; and (iv) register all image volumes to nanoscale precision. The pipeline ensures accurate ultrastructure preservation, permits independent optimization of SR and EM imaging modalities, and provides a comprehensive view of how specific subcellular components vary across the cellular volume.

RESULTS: Nearly every system we studied revealed unexpected results: intranuclear vesicles positive for a marker of the endoplasmic reticulum; peroxisomes of increasingly irregular morphology with increasing size; endolysosomal compartments of exceptionally diverse and convoluted morphology; a web-like adhesion network between cerebellar granule neurons; and classically EM-defined domains of heterochromatin and euchromatin each subcharacterized by the presence or absence of markers of transcriptional activity. Two-color cryo-SMLM enabled whole-cell image registration quantifiable down to ~40 nm accuracy. Cryo-SIM, even with its lower resolution, enabled unique discrimination between vesicles of like morphology and aided in segmenting complex 3D structures at FIB-SEM resolution within the crowded intracellular milieu.

CONCLUSION: Our pipeline serves as a powerful hypothesis generator to better understand the findings of biochemistry in the context of the spatially compartmentalized cell. Our approach also carefully preserves the native ultrastructure upon which such hypotheses are based, thus enabling cell-wide or cell-to-cell investigation of the natural variability in protein-ultrastructure relationships.
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Within cells, the spatial compartmentalization of thousands of distinct proteins serves a multitude of diverse biochemical needs. Correlative super-resolution (SR) fluorescence and electron microscopy (EM) can elucidate protein spatial relationships to global ultrastructure, but has suffered from tradeoffs of structure preservation, fluorescence retention, resolution, and field of view. We developed a platform for three-dimensional cryogenic SR and focused ion beam–milled block-face EM across entire vitreously frozen cells. The approach preserves ultrastructure while enabling independent SR and EM workflow optimization. We discovered unexpected protein-ultrastructure relationships in mammalian cells containing intranuclear vesicles containing endoplasmic reticulum–associated proteins, web-like adhesions between cultured neurons, and chromatin domains subclassified on the basis of transcriptional activity. Our findings illustrate the value of a comprehensive multimodal view of ultrastructural variability across whole cells.

Electron microscopy (EM) has revealed an intricate world inside eukaryotic cells (1), spatially organized at all length scales from nanometer-sized molecular assemblies to cell-spanning structures such as actin stress fibers and microtubules. However, even within different regions of the cell, there are notable differences in the structure of individual components, such as nuclear chromatin organization (2) or the morphology of the endoplasmic reticulum (ER), which is highly convoluted and compact in the perinuclear region, yet sparsely reticulated in lamellipodia (3). Thus, a comprehensive picture of cellular organization requires nanometer-level three-dimensional (3D) imaging of whole cells.

Although cryogenic EM (cryo-EM) tomography offers subnanometer 3D resolution (3), it is limited to sparse deposits of extracted macromolecules, cellular sections of submicrometer thickness (4–7), or thin lamella sculpted with cryo–focused ion beam (FIB) milling (8, 9). In contrast, serial FIB ablation and imaging of the exposed face of resin-embedded specimens by scanning electron microscopy (FIB-SEM) routinely achieves 8-nm isotropic 3D sampling (10–12), a degree of precision not possible with traditional 3D EM by diamond knife serial array (13, 14) or block-face sectioning (15). However, EM produces grayscale images in which the unambiguous identification and 3D segmentation of many subcellular structures can be challenging, and where the distributions of specific proteins can rarely be identified.

In response, correlative light and electron microscopy (CLEM) techniques have been developed that combine the global contrast and high resolution of EM with the molecular specificity of fluorescence microscopy (16, 17). With the advent of super-resolution (SR) microscopy (18), such techniques now offer a closer match in resolution between the two modalities (table S1 and text S1), allowing specific molecular components to be visualized at nanoscale resolution in the context of the crowded intracellular environment. However, SR/EM correlation often involves trade-offs in sample preparation among retention of fluorescent labels, sufficiently dense heavy metal staining for high-contrast EM, and faithful preservation of ultrastructure, particularly when chemical fixation is used (19–22).

Here, we describe a pipeline (fig. S1) for correlative cryo-SR/FIB-SEM imaging of whole cells designed to address these issues. Specifically, cryogenic, as opposed to room-temperature, SR performed after high-pressure freezing (HPF) allowed us to use a standard EM sample preparation protocol without compromise. We correlated cryogenic 3D structured illumination microscopy (SIM) and single-molecule localization microscopy (SMLM) SR image volumes, revealing protein-specific contrast with 3D FIB-SEM image volumes containing global contrast of subcellular ultrastructure. The SR

Movie 1. Raw single-molecule frames over time since initial illumination, illustrating dark-state conversion efficiency and background as functions of temperature and emission wavelength.

High-pressure–frozen U2OS cells expressing fluorescent protein or dye-labeled TOMM20 to mark the outer mitochondrial membrane are shown at 10 different intervals over 3.5 hours of illumination. Bright continuous emitters are fluorescent-bead fiducial markers. As seen, all six emitters exhibit better single-molecule contrast at ~8 K than at 77 K, yielding more accurate single-molecule localization (Fig. 1A to C, and fig. S9).
modality highlights features not readily apparent from the EM data alone, such as exceptionally long or convoluted endosomes, and permits unique classification of vesicles of similar morphology such as lysosomes, peroxisomes, and mitochondrial-derived vesicles.

Cell-wide 3D correlation also reveals unexpected localization patterns of proteins, including intranuclear vesicles positive for an ER marker, intricate web-like structures of adhesion proteins at cell-cell junctions, and heterogeneity in euchromatin or heterochromatin recruitment of transcriptionally associated histone H3.3 and heterochromatin protein 1α (HP1α) in the nuclei of neural progenitor cells as they transition into differentiated neurons. More generally, whole-cell cryo-SR/FIB-SEM can reveal compartmentalized proteins within known subcellular components, aid in the discovery of new subcellular components, and classify unknown EM morphologies and their roles in cell biology.

**Cryogenic SR below 10 K: Motivations and photophysical characterization**

To avoid artifacts associated with chemical fixation (fig. S2), our pipeline begins with cryo-fixation via HPF (23, 24) of whole cells cultured on sapphire disks 3 mm in diameter and 50 μm thick (text S2). Unlike plunge-freeze methods, HPF reliably freezes specimens up to 200 μm thick (21, 23, 25, 26) in their entirety within vitreous ice in milliseconds, providing an exact snapshot of subcellular ultrastructure (fig. S3 and movie S1). Each sapphire disk provides an optically flat and transparent back surface for aberration-free SR imaging, along with the high thermal conductivity needed to minimize specimen heating and potential ice recrystallization under the intense (~kW/cm²), long-lasting illumination used during SMLM. Frozen specimens are inspected, cleaned (movie S2), and loaded onto a solid copper sample holder (fig. S4) in a covered, liquid nitrogen (LN₂)-cooled preparation chamber (fig. S5) before transfer through a load lock to an evacuated optical cryostat modified for SR imaging (fig. S6).

Cryo-SR increases fluorophore photostability (27). This allowed us to achieve the high photon counts required for precise single-molecule localization, despite the modest numerical aperture (NA 0.85) we were compelled to use in order to image through the cryostat window, vacuum, and sapphire substrate (fig. S1 and text S9). This, along with a high collection efficiency of 405-nm illumination (28, 29), allowed us to acquire multicolor SIM/SMLM images of the same cells without substantial photobleaching. In turn, this enabled SIM/SMLM correlation in three or more colors (movie S3) and allowed us to quickly image and assess many cells across the substrate by 3D SIM. We could then concentrate on the best candidates for much slower, higher-resolution imaging by 3D SMLM.

Most cryo-SR systems to date operate with LN₂ cooling near 77 K (7, 27, 29–34). However, we opted for a liquid helium (LHe)-cooled microscope, which allowed us to explore photophysics at any temperature down to 8 K (text S4). In particular, we exploited a sharp increase in the lifetime of a dark state D₁ for many fluorophores with decreasing temperature (fig. S7) that allowed them to be shelved efficiently for long periods. Such shelving has important implications for SMLM because it dictates the dynamic contrast ratio (DCR), defined by the time a given molecule is OFF and shelved in the dark state normalized to the time it is ON and cycling between singlet states S₁ and S₂ (fig. S7) to emit light. Molecules with high DCR can be expressed at higher density, creating SMLM images of higher fidelity and resolution, with less chance of spontaneous overlap of the diffusion spots from multiple molecules that would otherwise hinder precise localization.

We measured (Fig. 1A) the DCR of six different fluorophores at both 8 K and 77 K from the OFF/ON blinking behavior of isolated single molecules (fig. S8 and text S4a). In addition, we compared (Fig. 1B) their static contrast ratios (SCR, defined by the ratio of their signal in the ON state to their local background; fig. S9), which must also be high for precise localization, during SMLM imaging of densely labeled mitochondria (Movie 1, fig. S9, and text S4b). DCR and SCR tended to increase with shorter emission wavelengths, making such fluorophores better suited to high-quality SMLM imaging (Fig. 1C). SCR also often improved at lower temperature (Fig. 1B). These trends are consistent with the photophysical argument that the dark-state lifetime should increase with increasing energy from D₁ to S₀ normalized to the thermal energy. In particular, we observed substantial gains in the SCR and DCR of JF525 (35) when operating with LHe, which, in conjunction with mEmerald, enabled high-quality two-color SMLM of densely labeled structures. However, if only cryo-SIM and/or single-color cryo-SMLM is needed, or if further study uncovers fluorophores spectrally distinct from mEmerald that work just as well at 77 K, then operation with LN₂ may prove sufficient.

To compare the relative merits of these labels for cryo-SMLM, we imaged two U2OS cells, targeting the ER membrane with mEmerald (green) and the mitochondrial outer membrane with Halo-JF525 (magenta) (36) or vice versa (Fig. 1D). Although both labels produced high-density, high-precision SMLM images of both targets, the Halo-JF525 images exhibited numerous bright puncta in both cases (Fig. 1D and fig. S10B). Although these may result from aggregation of Halo-tagged proteins, the presence of similar puncta in cryo-SMLM images of the ER obtained via SNAP (37) or CLIP (38) tag targeting of JF525 (fig. S10, C and D) suggest that they arise from a subset of extremely long-lived JF525 molecules that undergo numerous switching cycles. Indeed, the long persistence of JF525 and other labels at
8 K necessitated a fresh, data-driven approach (fig. S11 and text S5b) to the problem of correctly assigning and integrating the multiple photon bursts from each molecule. Even so, in all cases mEmerald yielded images probably more reflective of the true molecular distribution.

Cell-wide 3D correlation of cryo-SR with FIB-SEM
A key advantage of our pipeline is that inserting the cryo-SR step between cryofixation and freeze substitution/staining for FIB-SEM allowed us to decouple the sample preparation protocols for the two imaging modalities. This avoids the trade-offs of fluorescence retention, dense heavy metal staining, and ultrastructure preservation of resin embedding-based protocols (39–42). Furthermore, it allowed us to rapidly (~20 min) survey hundreds of cells across the sapphire disk (fig. S12A), so as to identify those of promising morphology and expression levels. We could then inspect these further at higher resolution by 3D cryo-SIM (5 min per cell per color) and select the very best ones of these for the ~1 to 2 days per cell per color required for 3D cryo-SMLM and ~10 to 15 days per cell needed for EM sample preparation and FIB-SEM imaging.

Thus, after cryo-SR imaging, we removed the frozen, disk-mounted specimens from the cryostat (fig. S12B) and processed them (text S6)....
via freeze substitution (19–21), heavy metal staining, and embedding in Eponate 12 resin (fig. S12C). After coarse trimming of the resin block and removal of the sapphire disk, we re-embedded the remaining tab in Durcupan, imaged it with x-rays (fig. S12D), and correlated this to the original disk-wide view (fig. S12E) to identify the region (typically 100 µm × 100 µm × 10 µm) containing the cells of interest imaged previously with cryo-SR. Additional micrometre trimming isolated this region (fig. S12, F to I), which we then imaged at 4- to 8-nm isotropic voxels in 3D by FIB-SEM (22).

To exploit the full potential of correlative microscopy, the different imaging modalities need to be mutually registered to the level of their spatial resolution. Given the high resolution of both cryo-SMLM and FIB-SEM, and our desire to extend their correlation across whole cells in 3D, registration to this level is challenging. For example, slight magnification differences or deviations from ideal flatfield and rectilinear imaging coupled with potentially nonuniform FIB milling increase registration errors quickly with increasing field of view. Furthermore, freeze substitution and resin embedding introduce nonlinear and spatially inhomogeneous sample deformations (arrows, Fig. 2A) (43) between the cryo-SR and FIB-SEM imaging steps that have a substantial nonlinear component requiring deformable registration to achieve alignment to this level of accuracy.

Taking advantage of our protocol, we densely labeled ubiquitous intracellular organelles such as the ER and mitochondria that could be readily identified in both the cryo-SMLM and FIB-SEM data and used them as landmarks (e.g., Fig. 2B and fig. S13) to register the EM to the SR across the cellular volume (text S7), using the software package BigWarp (44). To quantify the accuracy of this correlation, we independently measured the deformation fields $\mathbf{DF}_\text{ER}$ and $\mathbf{DF}_\text{mito}$ from only ER or mitochondrial landmarks, respectively, after aligning these color channels to one another using fluorescent bead fiducial markers.

The data also immediately illustrate the power of cryo-SR and FIB-SEM correlation. For example, clusters of ER3 seen by cryo-SMLM to dot ER tubules (orange arrows, Fig. 3B) might easily be dismissed as artifacts of labeling or fixation, but instead correlate (Fig. 3D) with varicosities in these tubules as seen by FIB-SEM (Fig. 3C). It is also immediately apparent by FIB-SEM that vesicles of various sizes are ubiquitous throughout the cell. However, these can come in many forms: peroxisomes, lysosomes, endosomes, or, as identified by our correlation, TOMM20-positive vesicles (red arrows, Fig. 3, H and J, and fig. S16). Given their small (~100 to 200 nm) size and proximity to mitochondria, these may represent mitochondria-derived vesicles (MDVs). MDVs are...
believed to play a key role in mitochondrial quality control by sequestering unfolded or oxidized mitochondrial proteins and transporting them to lysosomes or peroxisomes for degradation \((45)\). There remain, however, many questions about what proteins regulate these processes and where they are distributed.

Our data also revealed three instances of intranuclear vesicles, again ~100 to 200 nm in size, positive for the ER lumen protein ER3 (Fig. 3A, left inset orthoslices; fig. S15, correlation examples 119 and 164). In dividing somatic cells, the nuclear membrane breaks down at prometaphase and its proteins are dispersed within the ER, which remains continuous throughout mitosis \((46)\). The nuclear membrane then begins to reassemble in anaphase when ER-like cisternae contact the chromatids of the daughter cells, and nuclear membrane proteins become immobilized there \((46–49)\). Thus, one possibility is that ER lumen-positive intranuclear vesicles in interphase are the remnants of such contacts that do not completely return to the extranuclear ER after the nuclear membrane is fully reestablished. Alternatively, a small fraction of the total ER volume might be disrupted into vesicles during its rearrangement in mitosis and become similarly trapped when the nuclear membrane reforms.

Another important class of vesicles in cells are peroxisomes, which catabolize long-chain fatty acids via \(\beta\)-oxidation and reduce reactive oxygen species such as hydrogen peroxide \((50)\). Peroxisomes can adopt a variety of sizes, shapes, and distributions depending on cell type and environment \((50, 51)\). Accurately capturing these morphologies and their spatial relationship to other organelles can be difficult with traditional chemical fixation and EM staining protocols \((52, 53)\). Furthermore, serial section transmission EM or mechanically sectioned block-face EM \((54)\) lack the axial resolution to precisely measure morphological parameters at the sub–100-nm level, whereas cryo-FIB-SEM \((55)\) or cryo-EM tomography lacks the field of view to explore more than a small fraction of the total cellular volume.

We used cryo-SMLM/FIB-SEM to image and semi-automatically segment (text S8) 466 mature peroxisomes across two entire vitrinely frozen HeLa cells expressing mEmerald tagged to the peroxisomal targeting sequence SKL, and Halo/JF525-TOMM20 to mark mitochondria (Fig. 4, Movie 3, and fig. S17). Independent two-channel SR/EM registration revealed a correlation accuracy (median \(e\)) of 8 5nm (fig. S14, C and D). Peroxisomes with volumes smaller than 0.01 \(\mu m^3\) always assumed nearly spherical shapes, presumably to minimize their surface area under the influence of surface tension (e.g., Fig. 4, A and H). Increasingly irregular shapes such as plates (Fig. 4B), cups
(Fig. 4C), or hollow spheres (Fig. 4D) formed with increasing volume (fig. S17, lower rows). This may serve to regulate reaction kinetics within peroxisomes (56). Some irregularly shaped peroxisomes were in close proximity to other organelles or as part of multi-organelle assemblies (Fig. 4, E to G), consistent with 3D observations in live cells (57). These assemblies may facilitate the transfer of cargo between organelles responsible for distinct and possibly incompatible biochemical processes (57), such as the sequential breakdown of fatty acids between peroxisomes and mitochondria (58).

Finally, we explored the endolysosomal pathway, the compartments of which are notoriously sensitive to artifacts of fixation or protein overexpression (59–61). We used correlative cryo-SIM/FIB-SEM to image transferrin (Tfn)–containing endolysosomal compartments in a SUM-159 cell previously incubated for 30 min in media containing Alexa Fluor 647–conjugated Tfn (Fig. 5A and beginning of Movie 4). The density of labeled compartments was low enough to assign each discrete SIM feature (Fig. 5A, inset) to a single structure as seen by FIB-SEM, and then render each such compartment with 8-nm isotropic voxels. Despite its much lower resolution, SIM was essential to identify which compartments in the FIB-SEM data represented endolysosomes and to spot the many such structures of extremely convoluted morphology in the crowded intracellular environment that were not readily apparent by FIB-SEM alone. These included elongated tubules (Fig. 5, B to E, magenta) that likely represent recycling endosomes, highly corrugated endosomes (Fig. 5, B and D, right), and early endosomes with protruding tubules of 50 nm width possibly associated with retromers (62) of sub–50 nm width. Given that cryo-SIM is much faster than cryo-SMLM and can use a wide variety of spectrally distinct labels, it can be a broadly useful tool in its own right to guide the 3D segmentation of dense FIB-SEM data and ensure the correct identification of specific subcellular features.

**Movie 4. Cryo-SIM/FIB-SEM reveals the morphological heterogeneity of the endolysosomal system.** A correlative dataset of a SUM-159 cell after endosomal uptake of Alexa Fluor–conjugated transferrin is shown. Part 1: 3D cryo-SIM data (green), correlative orthoslices, and correlative volume render (cyan, plasma membrane; orange, cellular interior). Part 2: ~13-μm³ subvolume showing segmentations of all transferrin-containing compartments. Part 3: Same, but for a different ~20-μm³ subvolume (Fig. 5).

**Molecular underpinnings of ultrastructural specialization in neuronal cell-to-cell adhesions**

Cell-to-cell adhesions mediate cell migration, nucleate cell polarity, and spur communication between individual cells...
in multicellular organisms (63, 64). Although the molecular context and ultrastructure of cellular adhesions to rigid artificial substrates are well characterized (65, 66) those between cells in complex 3D environments are not. Neuronal adhesions are crucial for brain development, playing an integral role in sorting neurons according to their maturation status (67, 68), forming the laminar structure of the brain (69, 70), and ultimately promoting the complex neuronal interactions that drive circuit morphogenesis (77). However, they have been difficult to study because they are disrupted by chemical fixation (19-21) and because 3D geometries of neuronal contacts require isotropic 3D-EM and high-resolution LM.

We used cryo-SIM to visualize transiently expressed junctional adhesion molecule (JAM)-C (67, 69, 72), a tight-junction component, fused to JF549i-conjugated SNAP (35, 73), and 2x-mVenus-drebrin, a cytoplasmic actin-microtubule cross-linker protein (74), in cryofixed mouse cerebellar granule neurons (CGNs) (Fig. 6A and Movie 5). The JAM-C-defined adhesion between two labeled somas was not uniform at their shared membrane contact zone (Fig. 6B) but formed a web-like structure, with drebrin preferentially associated with the edges of the JAM-C regions.

To determine whether these protein distributions correlated with membrane ultrastructure at the contact zone, we imaged the same cells by FIB-SEM (Fig. 6C). The density of heavy metal staining at the plasma membrane was also nonuniform (Fig. 6D), with the densest staining correlating perfectly with JAM-C (compare Fig. 6, B, D, and G). Moreover, the densely stained plasma membrane was less curved than the electron-lucent plasma membrane. To quantify this, we segmented the plasma membrane within the contact zone into regions of high and low electron density (Fig. 6, F and I) and then calculated the curvedness (text S9) in each (Fig. 6, H and I). The low-density plasma membrane had a curvedness of 11.3 μm⁻¹, whereas the high-density, JAM-C-rich plasma membrane had a curvedness of 5.0 μm⁻¹.

Although the smooth nature of the adhesion as defined by JAM-C is expected because of the mechanical tension induced by the juxtacrine interaction (75-77), the fact that the adhesion does not comprise the whole contact area between these two cells is unexpected. Furthermore, the enrichment of drebrin in the regions adjacent to JAM-C contrasts with the laminar stacking of adhesion-associated cytoskeletal adapter proteins found in focal or cadherin-based adhesions on glass (65, 66).

Chromatin domains and their reorganization during neuronal differentiation

In addition to adhesion, CGNs provide an excellent model system to study the cell biological underpinnings of neural development, owing to their strongly stereotyped developmental programs as they differentiate from cerebellar granule neuron progenitors (GNPs) (78). Intrinsic to this process is the 3D structural reorganization of their nuclear chromatin domains (79, 80). To explore this in detail, we first used 3D live-cell lattice light sheet microscopy (LLSM) (81). Flow-sorted GNPs expressing the EGFP-Atoh1 marker of the GNP state (82, 83) possessed nuclei that were significantly larger than those of terminally differentiated CGNs (Fig. 7, A and B, and text S10). Moreover, longitudinal LLSM live imaging revealed that GNPs rapidly condense their nuclei to the size of CGNs while Atoh1-EGFP expression fades (Fig. 7, A and B, and movie S4).

To uncover the intricate 3D transformations in nuclear architecture that accompany nuclear condensation during GNP differentiation, we then applied cryo-SIM to image a
cohort of seven GNPs and nine CGNs. These collectively contained >2000 μm³ of two nuclear domain reference proteins: mEmerald-tagged heterochromatin protein 1a (HP1α), a prototypical heterochromatin marker (84), and JF525-conjugated SNAP-histone 3.3 (H3.3), a replacement histone subunit that is loaded on transcriptionally active nucleosomes (85) (Fig. 7, C and G, top, and fig. S18). We followed this with FIB-SEM imaging and segmentation of the resulting data (86) according to the classic EM definitions of compacted heterochromatin, open euchromatin, and nucleoli (2) (Fig. 7, C and G, bottom). GNP and CGN nuclei possessed similar total nuclear volumes of compacted heterochromatin (GNP = 47 ± 2 μm³, CGN = 45 ± 3 μm³); however, GNP nuclei had a significantly higher total nuclear volume of euchromatin (GNP = 84 ± 8 μm³, CGN = 61 ± 6 μm³) that accounted for a significant fraction of the size differential with CGNs (Fig. 7K).

Registering the cryo-SIM data onto the FIB-SEM results (Fig. 7, D to F and H to J, Movie 6, figs. S19 to S21, movie S5, and text S11) allowed us to subclassify these classical EM chromatin domains according to their correlation to HP1α or H3.3 (figs. S22 and S23 and text S12). Such correlation revealed variations in these chromatin domains linked to neuronal differentiation that cannot be discerned by ultrastructure alone, including classical compacted heterochromatin domains with alternating layers HP1α and H3.3 (Fig. 7J). Indeed, although FIB-SEM showed little difference in the absolute volume of compacted heterochromatin before and after differentiation, correlation with cryo-SIM revealed that CGN nuclei had ~50% more normalized nuclear volume of HP1α-loaded heterochromatin than did GNPs (GNP = 8 ± 1%, CGN = 12 ± 2%; Fig. 7L). Moreover, measurements of surface area versus volume showed that HP1α-loaded heterochromatin became substantially more compact during nuclear condensation (fig. S23A).

Fig. 6. Membrane proteins correlate to membrane ultrastructure at cell-cell adhesions. (A) Cryo-SIM volume of cultured mouse cerebellar granule neurons transiently expressing JF549i/SNAP-JAM-C (green) and 2x-mVenus-drebrin (magenta). (B) MIP through a slab ~3 μm thick [white box in (A)] centered on the contact zone between two cell bodies. (C) FIB-SEM volume of the same region in (A), with plasma membrane (cyan), intracellular content (orange), and segmented electron-dense regions of the contact zone (white). (D) FIB-SEM MIP through the same region in (B), after masking the nuclei. (E) Single FIB-SEM slice through the contact zone at the central vertical line in (D). (F) Same as (E), with more (blue) and less (red) electron-dense membranes traced. (G) Same as (E) overlaid with the JAM-C signal. Scale bar, 500 nm. (H) Histograms of the curvedness (text S9) for the membrane regions with high (blue) and low (red) electron density. (I) Partial segmentation of the cells’ membranes in the contact zone, color-coded according to curvedness, with brighter colors indicating larger values. Note the high correlation among JAM-C (B), electron density (D), and membrane curvedness (I) (Movie 5). White box dimensions: 9.5 μm × 4.7 μm × 1.1 μm.
similar amounts of H3.3-loaded euchromatin nuclei. Although both GNPs and CGNs had substantial differences between GNP and CGN chromatin and euchromatin also revealed sub-

Analysis of H3.3 relationships to heterochromatin and euchromatin also revealed substantial differences between GNP and CGN nuclei. Although both GNPs and CGNs had similar amounts of H3.3-loaded euchromatin (GNP = 27 ± 4%, CGN = 32 ± 3%; Fig. 7L) indicative of transcriptionally active regions, GNPs had 50% more normalized nuclear volume of a H3.3-free form of euchromatin than did CGNs (GNP = 29 ± 4%, CGN = 20 ± 2%; Fig. 7L). Live-cell LLSM operating in the higher-resolution SIM mode revealed that these large H3.3-free voids in GNP nuclei contain mEmerald-CMA, a marker of H3K27me3 and H3Kme3-loaded poised chromatin (87), which suggests that groups of poised genes are organized in a region-specific fashion in neural progenitors (fig. S24, movie S6, and text S13).

GNP differentiation into CGNs also resulted in the unexpected accumulation of H3.3 in heterochromatin, nearly twice as abundant in CGNs as in GNPs (GNP = 13 ± 1%, CGN = 22 ± 3%; Fig. 7L). Like classical HP1α-loaded heterochromatin, H3.3-loaded heterochromatin also underwent compaction during CGN differentiation (fig. S23C). The presence of a large fraction of H3.3-loaded heterochromatin in differentiated neurons was surprising, given that H3.3-loaded heterochromatin species are abundant in pluripotent embryonic stem cells (ESCs) but have not been observed in most of their somatic cell derivatives (88, 89). Furthermore, LLS-SIM revealed that H3.3-loaded heterochromatin is likely not due to H3.3 recruitment to telomeres or centromeres, as has been reported for ESCs (fig. S24B).

Finally, heterochromatin subdomains exhibited spatially distinct organization patterns depending on whether they were loaded with HP1α or H3.3 (fig. S22A and movie S7). Additional analysis based on the density of heavy metal staining in a correlated 4-nm FIB-SEM dataset revealed that H3.3-loaded heterochromatin was less densely packed than HP1α-loaded heterochromatin in CGN nuclei; hence, molecularly defined heterochromatin subdomains are not only spatially distinct at the level of the whole nucleus but are also morphologically distinct at the ultrastructural level (fig. S22B).

**Discussion**

Much of what we know about the structural and functional organization of the cell at the nanoscale comes from a synthesis of the findings of EM, biochemistry, and molecular biology. Although this synthesis has proved powerful, fusing the insights from these disparate methods necessarily involves developing models, and therefore possible biases, of how specific proteins are spatially distributed in relation to the EM ultrastructure that bear closer examination. Correlative cryo-SR/FIB-SEM enables such examination by combining two complementary datasets, often revealing unanticipated protein localization patterns or ultrastructural morphologies at variance with such models. At the same time, the approach enables the discovery of new subcategories of functionally distinct subcellular structures that appear morphologically similar when using either SR or EM alone. As such, it provides observations upon which more refined models can be developed in a
Movie 5. Correlative cryo-SIM/FIB-SEM reveals a web-like adhesion pattern between adjacent cerebellar granule neurons. 
Part 1: Cryo-SIM and FIB-SEM volume renderings of a field of CGNs expressing adhesion proteins JAM-C (green) and drebrin (magenta). Part 2: Correlation between electron density at the plasma membrane, JAM-C cryo-SIM signal, and plasma membrane curvature at the interface between two CGNs (Fig. 6).

Movie 6. Chromatin compaction during differentiation and identification of novel chromatin subdomains. Correlative datasets of granule neuron progenitor (GNP, left) and cerebellar granule neuron (CGN, right) are represented. Part 1: Overall correlation between the FIB-SEM (cyan, plasma membrane; orange, cellular interior) and cryo-SIM of the nuclear domain reference proteins HP1α (green) and H3.3 (magenta). Part 2: Cutaway views of EM-defined chromatin domains for a GNP nucleus (left) and a CGN nucleus (right). Part 3: Orthoslices through the CLEM volumes indicating subdomains defined by overlap between EM-defined nuclear domains and nuclear domain reference proteins. Part 4: 3D surface renderings of CLEM-defined nuclear chromatin subdomains for the GNP and CGN nuclei (Fig. 7).

Way mutually consistent with the findings of SR, EM, live imaging, and biochemistry. Of course, the value of cryo-SR/FIB-SEM to this enterprise depends on the extent to which it reveals the native ultrastructure of the cells it images, and the extent to which these cells are representative of the normal physiological state of their class. We designed our pipeline with these goals in mind. HPF immediately followed by cryo-SIM imaging of cells in vitreous ice without any intervening chemical modification ensures that a faithful, unperturbed snapshot of the cell is captured, and allows SR and EM sample preparation protocols to be decoupled and independently optimized. Wide-field cryo-fluorescence imaging to rapidly survey hundreds of cells, followed by higher-resolution inspection of likely candidates by multicolor 3D cryo-SIM at a few minutes per cell, ensures that only those cells of physiological morphology, or cells in a specific desired physiological state [e.g., (90)], are considered for time-intensive cryo-SMLM and FIB-SEM. Lastly, freeze substitution provides excellent preservation of native ultrastructural detail while subsequent whole-cell 3D FIB-SEM gives a comprehensive picture of subcellular components across all regions of the cell, at 4- or 8-nm isotropic voxels not possible with serial-section transmission EM or mechanically sectioned serial block-face EM.

That being said, cryo-EM tomography of thin lamellae excavated from whole cells by cryo-FIB (8, 9) offers molecular resolution without any risk of ultrastructural perturbation by heavy metal staining and resin embedding. Given, however, that the lamellar volume is typically only a small fraction of the entire cellular volume, many structures of interest will be missed entirely, and those that are seen may not exhibit the same morphology as in other regions of the cell. Thus, FIB-SEM and cryo-EM tomography are complementary, and developing a pipeline to do both in conjunction with cryo-SR would be a worthwhile endeavor.

Indeed, the unique ability of FIB-SEM to image whole cells and tissues at 4- to 8-nm isotropic voxels over volumes as large as $10^3 \mu m^3$ makes it an ideal tool to map in toto the 3D ultrastructural relationships in living systems. However, to unlock its full potential, robust automated identification and segmentation of specific intracellular features of interest are required, ideally in relationship to neighboring structures with which such features might interact. This remains challenging to accomplish at scale, given the magnitude of the data involved [e.g., 100 GB in Fig. 7 and 19.5 TB in (22)]; the diversity, spatial density, and conformational complexity of intracellular compartments; and the monochromatic nature of the data. Cryo-SR can play an important role in the development of scalable segmentation, both in the validation of training sets for machine learning and in confirmation of the resulting segmented outputs.

We can also envision a number of possible improvements to our pipeline. First, live-cell imaging immediately prior to freezing would allow correlation of dynamics to ultrastructure (67), refine selection to cells of physiological behavior, and enable pharmacological, optogenetic, or other perturbations to be applied. However, the logistics for rapid and noninvasive transition from live imaging to the frozen state will require substantial technological development. Second, an extension of cryo-SR/FIB-SEM to specimens such as small gene-edited organisms or organoids that are more physiologically relevant than the isolated adherent cells with ectopically expressed markers presented here should be feasible within the $200-\mu m$ thickness limit for HPF by incorporating adaptive optics for aberration-free deep imaging. Third, the axial resolution of both cryo-SIM and cryo-SMLM could be improved by a factor of ~5 to 10 by designing a dual-window cryostat that uses opposed objectives and coherent detection, such as in I5S (91) and iPALM (92). A next-generation pipeline combining these improvements could prove an even more powerful discovery platform to link 3D subcellular dynamic processes in cells, small whole organisms, and acute tissue sections to the nanoscale spatial distribution of the proteins driving these processes, all in the context of the global intracellular ultrastructure. However, even in its current form, our cryo-SR/FIB-SEM system can address a broad range of biological questions and is available to outside users wanting to do so (93).

**Materials and methods**

**Preparation of vitrified samples**

Specimens were cultured on 3-mm-diameter, 50-\mu m-thick sapphire dishes (Nanjing Co-Energy Optical Crystal Co. Ltd., custom order; text S2) before cryofixation with a Wohlgend Compact 2 high-pressure freezer. See text S4 for sample-specific protocols and plasmid maps.

**Cryogenic light microscopy**

To optically image vitrified samples at diffraction-limited resolution and beyond, they must be maintained below 125 K to avoid devitrification (94) and present a clean, optically flat surface for aberration-free imaging. To achieve these ends, we built our microscope around a modified commercial liquid helium continuous-flow cryostat (Janis Research Company, ST-500; fig. S6 and text S3) and imaged cells plated on sapphire coverslips (text S2) through the opposite surface, after clearing this surface of residual ice in a custom cryo-preparation chamber (fig. S5, movie S2, and text S2). We transferred samples from cold storage to the imaging cryostat using custom tools and procedures.
adapted to a commercial cryogenic vacuum transfer system (Quorum Technologies, PP3010T; fig. S6 and text S3). SIM images were processed as described (95); SMLM processing is described in text S15.

**EM sample preparation**

After optical imaging, samples were transferred back to cryo-storage before being freeze-substituted, resin-embedded, and re-embedded (text S6b and S6c). Desired regions of interest (ROIs) were identified in the plasticized specimens (fig. S12) using an XRadia 510 Versa micro X-Ray system (Carl Zeiss X-ray Microscopy Inc.) and then trimmed to expose small (≈100 µm × 100 µm × 60 µm) stubs (text S6d).

**FIB-SEM imaging**

Standard (8 nm × 8 nm × 8 nm isotropic voxel) FIB-SEM datasets were generated using a customized Zeiss Merlin crossbeam system (12) and further modified as specified in text S16. The SEM image stacks were acquired at 500 kHz per pixel with an 8-nm xy-z pixel using a 2-nA electron beam at 1.2-kV landing energy for imaging and a 15-nA gallium ion beam at 30 kV for FIB milling. Similarly, 4 nm × 4 nm × 4 nm voxel datasets were generated using a customized Zeiss GeminiSEM 500-Capella crossbeam system. The block face was imaged by a 250-pA electron beam with 0.9-kV landing energy at 200 kHz. The final image stacks were registered using a SIFT-based algorithm (96).

**Computing resources**

For most of the data analysis, except initial SMLM peak detection and fitting, we used a stand-alone Windows 10 x64 workstation with dual Xeon Gold 5122 CPUs (3.60 GHz) and 1 TB of RAM. For SMLM peak detection and fitting, we used up to 256 nodes on the Janelia cluster.

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Correlative three-dimensional super-resolution and block-face electron microscopy of whole vitreously frozen cells

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Visualizing whole cells at many scales
Cells need to compartmentalize thousands of distinct proteins, but the nanoscale spatial relationship of many proteins to overall intracellular ultrastructure remains poorly understood. Correlated light and electron microscopy approaches can help. Hoffman et al. combined cryogenic super-resolution fluorescence microscopy and focused ion beam milling scanning electron microscopy to visualize protein-ultrastructure relationships in three dimensions across whole cells. The fusion of the two imaging modalities enabled identification and three-dimensional segmentation of morphologically complex structures within the crowded intracellular environment. The researchers observed unexpected relationships within a variety of cell types, including a web-like protein adhesion network between juxtaposed cerebellar granule neurons.

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