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Think globally, act locally: Redefining organellar membrane environments through cryo-electron tomography

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Early enthusiasm for the "cellular revolution" in cryo-electron tomography (cryo-ET) was largely driven by the promise of resolving protein structures in native environments. However, a parallel trajectory has emerged that centers less on structure determination and instead on quantitative information about the nanoscale organization of organelle membranes and associated macromolecules. Together, these advances have shifted the field from descriptive visualization toward conceptual insight, and in the process, redefined what "local" means in organelle biology. In this review, I highlight recent studies that show how cryo-ET has illuminated specialized organelle membrane states defined by geometry, bilayer properties, protein patterning, and molecular sociology. These innovations reveal insights into membrane microenvironments and further establish cryo-ET as a bridge between structural and cell biology.

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Introduction

Organelles play important roles in eukaryotic cells by maintaining subcellular compartmentalization while simultaneously serving as functional sites for communication, signaling, and metabolism [1]. From micrographs collected by pioneering cell biologist George E. Palade over seven decades ago, we have long appreciated that organellar membranes are not uniform but rather spatially restricted environments that dictate organelle function [2,3]. Global changes in lipid and protein composition are established determinants of specialized membranes; however, far less is known about how specialization is established at the molecular level. While conventional electron tomography of resin-embedded specimens can resolve features such as membrane distance and global curvature, fixative artifacts and axial-resolution constraints impose a practical limit on the precision and localization of these measurements at the molecular scale [4,5]. On the other hand, structural methods such as single-particle cryo-EM provide an atomistic view of membrane protein structure but often lack a complete cellular context [6], limiting their application to organellar biology since many processes intrinsic to organelle lifecycles cannot be fully recapitulated *in vitro*. Together, these limitations leave a fundamental gap between what can be visualized in the cellular context and what can be structurally resolved to understand organellar membrane specialization at the molecular level.

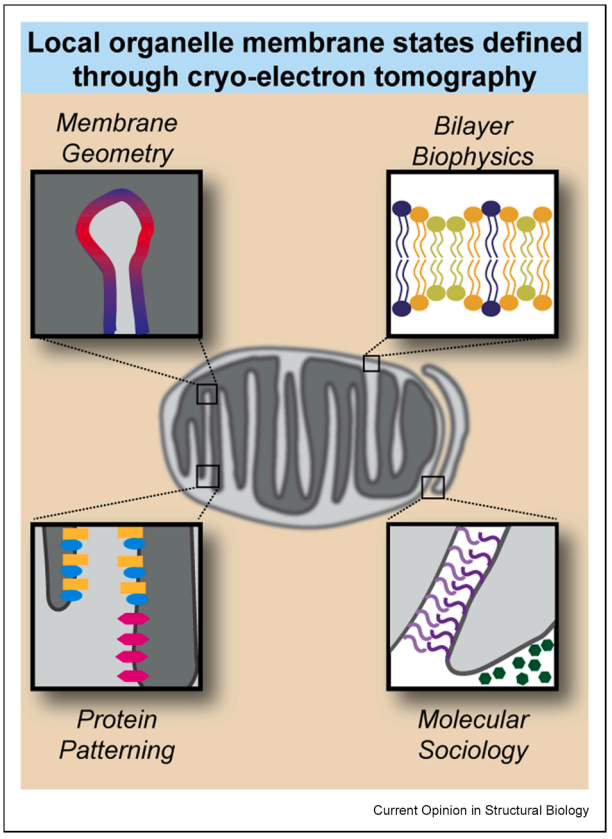
Cryo-electron tomography (cryo-ET) uniquely fills this niche by enabling visualization of proteins and lipid bilayers in cells at molecular resolution, thereby bridging conventional electron tomography and high-resolution protein structure determination methods. Early on, the ability to generate detailed three-dimensional reconstructions (i.e., tomograms) of the pristinely preserved (i.e., vitrified) cellular milieu captured the attention of both structural and cell biologists [7]. However, as the field matured, important questions, particularly from the cell biology community, were raised: *were these tomograms offering fundamentally new biological insight, or simply a more detailed version of the images captured by Palade and others?*

Several recent advances have shifted this balance, building on the foundation laid by landmark discoveries from decades of prior cryo-ET studies [8]. The application of cryo-focused ion beam (cryo-FIB) milling as an upstream sample preparation step enabled cryo-ET to move beyond imaging only the thinnest, peripheral regions of eukaryotic cells and into the crowded, perinuclear interior, providing access to a broader range of organellar biology [9]. Coupling this with automated

cryo-FIB milling and improved data acquisition schemes makes it feasible to collect hundreds to thousands of tomograms within a single sample preparation [10,11]. This increased scale translates into larger sample sizes that facilitate protein structure determination at higher resolutions, while also providing sufficient sample size to distinguish true membrane ultrastructural differences from the inherent heterogeneity of organelle populations. However, this scale also creates new challenges in rapidly extracting meaningful biological information from these information-rich tomograms. This has led to a wave of innovations in data analysis, including new tools for both protein structure determination and quantitative analysis of membrane ultrastructure (reviewed in Refs. [12–14]). Even when specific macromolecules cannot (yet) be resolved within crowded environments, correlative approaches (e.g., cryo-fluorescence microscopy) provide a means to interpret membrane structure in the context of specific, molecular labels [15].

With these capabilities in place, the question has shifted from "*what do we see?*" to "*what have we learned?*". A growing body of recent work shows that one of the clearest insights to emerge from cryo-ET studies is that "local" membrane environments are not defined simply by individual proteins, but by collective membrane states arising from geometry, bilayer biophysics, protein patterning, and molecular sociology (Figure 1). Importantly, while other techniques may provide information on any one of these features in isolation, cryo-ET uniquely enables each to be characterized within the same native cellular context, and increasingly, within the same dataset. In this review, I highlight recent studies that illustrate how cryo-ET is transforming our understanding of local organelle membrane organization and its links to cellular physiology.

Figure 1



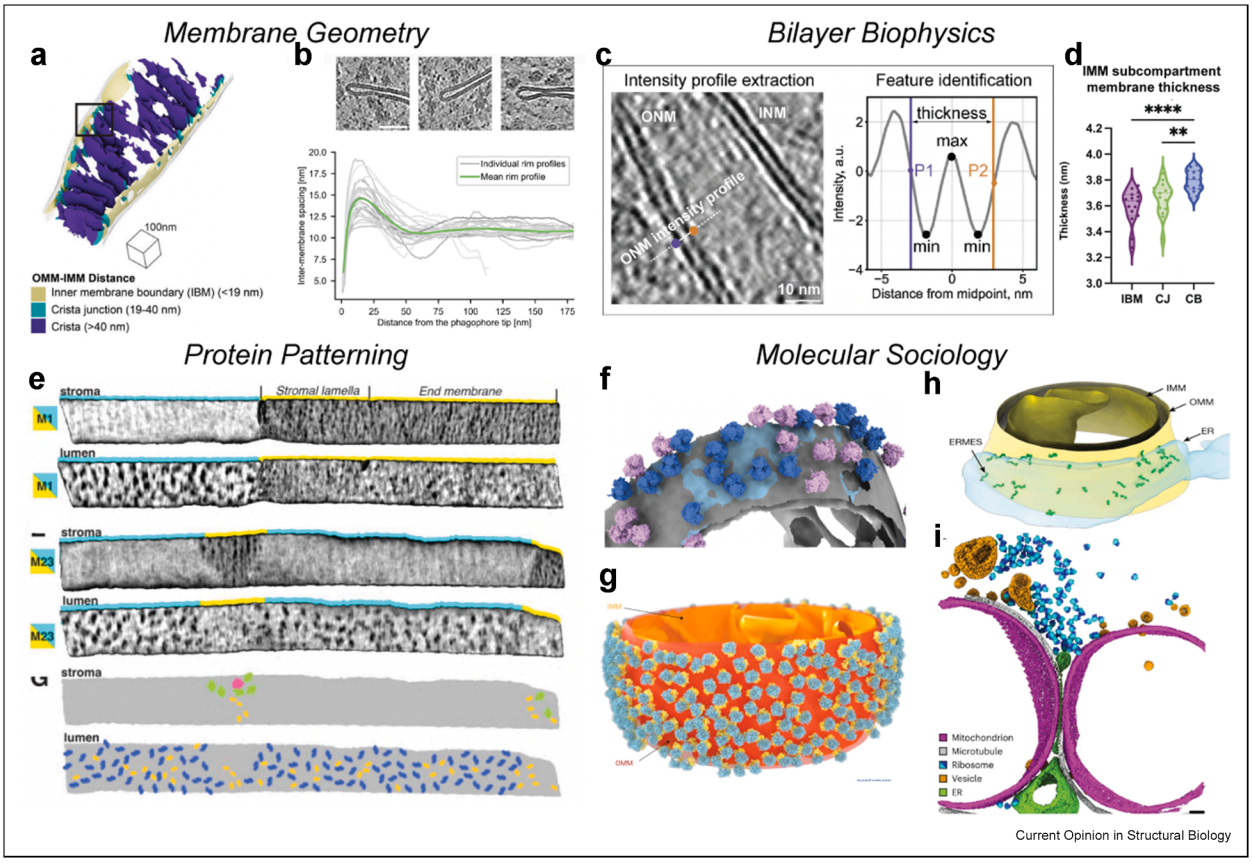
Local organelle membrane characteristics revealed by cryo-electron tomography (cryo-ET). This figure illustrates specialized organelle membrane states revealed by recent cryo-ET studies, defined by geometry, bilayer biophysics, protein patterning, and molecular sociology. While individual structural and cellular imaging approaches may provide information on any one of these features in isolation, cryo-ET uniquely enables each to be characterized within the same native cellular environment.

Membrane geometry defines organelle dynamics and function

Membrane shape has long been recognized as a hallmark of organelle identity, and for decades, changes in organellar membrane ultrastructure, visualized by room-temperature electron microscopy and other conventional imaging methods, have served as qualitative indicators of altered or pathogenic conditions. Modern cellular cryo-ET workflows sharpen these classic observations by transforming our understanding of membrane shape from a qualitative descriptor into a quantitative structural state, enabling detection of nanometer-scale differences in membrane ultrastructure across organelle populations. This is possible through software developments that enable robust extraction of membrane models (i.e., voxel segmentations and mesh surfaces) and the calculation of membrane ultrastructure, including membrane spacing, curvature, and orientation [16–18]. Mapping these measurements directly onto segmented membrane models reveals spatial "hotspots" of local geometry. An intuitive example of geometry-defined environments is the inner mitochondrial membrane, which can be automatically partitioned into its functionally distinct subcompartments (i.e., inner boundary membrane (IBM), cristae junction (CJ), cristae body (CB)) based on calculations of the distance between the inner and outer mitochondrial membranes (IMM and OMM, respectively) (Figure 2a).

These quantitative approaches also enable systematic comparison of membrane geometry across experimental groups to identify statistically significant changes that would be impossible to detect by visual inspection alone. Using these approaches, we showed that mitochondria undergoing adaptive network elongation in response to cellular stress exhibited coordinated remodeling of IMM geometry relative to mitochondria under basal conditions

Figure 2



Representative results from different cryo-ET studies that reveal specialized organelle membrane states. *Membrane Geometry:* **(a)** A membrane surface reconstruction of the inner mitochondrial membrane showing partitioning of spatially and functionally distinct subcompartments, as defined by the distance between the outer and inner mitochondrial membranes (OMM and IMM, respectively) [16]. **(b)** Representative tomogram slices of phagophore rims, scale bar: 50 nm (*upper panel*). The mean value (green) and individual profiles (gray) of inter-membrane spacing along the length of the phagophore show the phagophore tip dilation as a defining geometric feature of phagophore biogenesis during autophagy (*lower panel*) [21]. *Bilayer Biophysics:* **(c)** Tomogram slice showing the visual distinction of two lipid head groups (purple and orange points) within the outer nuclear membrane (ONM) (*left panel*). A membrane intensity profile extracted in three-dimensions along vectors extending from the purple and orange points is used to calculate lipid bilayer thickness (*right panel*) [28]. **(d)** A violin plot where each point represents the mean thickness per tomogram shows statistically significant variations between the inner boundary membrane (IBM), cristae junction (CJ), and cristae body (CB) subcompartments of the IMM [27]. *Protein Patterning:* **(e)** Flattened representations of membrane segments extracted from the tomogram (i.e., "membranograms") show lateral heterogeneity of the chloroplast photosystems between different appressed and non-appressed thylakoid membranes [38]. *Molecular Sociology:* **(f)** A membrane surface reconstruction of the OMM colored by OMM-IMM distance, with regions <10 nm shown in light blue. Ribosomes with their peptide exit tunnel near the OMM (i.e., oriented for import) (blue) show a higher degree of clustering in these regions relative to those not oriented for import (pink) [42]. (**g**) A segmentation model of the IMM (orange) and OMM (red). Ribosomes with their small subunit (yellow) facing toward and their large subunit (blue) facing away from the OMM (i.e., oriented for "hibernation") show clustering into arrays of trimers, tetramers, and pentamers [43]. **(h)** A segmentation model of the IMM and OMM (yellow) near the ER (transparent blue). Both long and short forms of the ERMES complex (green) exhibit a random distribution at this organelle membrane contact site [44]. **(i)** A segmentation model of a tomogram that overlaps with EGFP-FMRP fluorescence showing the molecular sociology of two mitochondria (presumed to be products of a recent fission event) surrounded by ER tubules and large clusters of ribosomes arranged in an organization that resembles polysomes near the OMM [47].

[16]. Because these measurements are performed on surface meshes (subsampling the voxel size) and aggregated across entire membrane surfaces (comprising hundreds of thousands of triangular elements), this approach enables the detection of statistically significant shifts in the distribution of OMM-IMM distances, including differences on the order of ∼0.8 nm. This difference is small, but *is it relevant?* Given that some IMM-localized lipid synthesis and transfer proteins can act in *trans* [19], changes in OMM-IMM spacing may regulate their activity and help explain stress-responsive alterations in OMM lipid composition that lead to adaptive network elongation [20]. Future work will be needed to directly test this proposed mechanism.

Geometry-defined states are also central to autophagy, a process that relies on dynamic membrane remodeling to ensnare cellular cargo for recycling. Distinct phases of phagophore biogenesis during macroautophagy in yeast are characterized by specific membrane geometries, with the inter-membrane spacing of the phagophore 'rim' structures exhibiting larger inter-membrane spacing (~14 nm) relative to the phagophore body (~11 nm) [21] (Figure 2b). Interestingly, these geometries seem to be specific to the type of cargo being engulfed, as the autophagy associated with the selective clearance of pathogens (i.e., xenophagy) in mammalian cells has comparatively larger intermembrane spacing for the rim (~29 nm) and body (~13 nm) [22]. Snapshots of mitochondria-specific autophagy (i.e., mitophagy) revealed phagophore rim membrane spacing that appeared even larger (~40 nm, estimated from micrographs) [23].

Phagophore remodeling in mitophagy is also accompanied by pronounced remodeling of the IMM, such as a twofold decrease in detectable cristae per mitochondrial volume and significant increases in the cristae surface area-to-volume ratio on the order of 0.4 nm^{-1}. These 'small' detectable changes in cristae geometry led to large impacts on the distribution of key mitochondrial complexes, such as ATP synthase, causing them to relocalize from highly curved CB regions to mostly planar IBM regions. This is significant because many cryo-ET studies spanning over a decade have demonstrated that the assembly of ATP synthase into dimers stabilizes regions of high membrane curvature and provides a structural basis for cristae formation and efficient oxidative phosphorylation [24]. Beyond ATP synthase, proteins such as OPA1 also regulate cristae spacing and connectivity in an isoform- and expression-dependent manner [25], and such alterations can impact respiration. Collectively, these studies establish that nanometer-scale changes in membrane geometry can significantly affect organelle function and dynamics.

Lipid bilayer thickness defines membrane microdomains

Although lipid heterogeneity has long been inferred, directly detecting localized differences in organelle bilayer composition within cells has remained challenging. Under low-defocus conditions, phospholipid bilayers appear as two dark, roughly parallel lines in cryo-ET data, corresponding to the headgroup-rich regions of each leaflet (Figure 2c). We and others have used segmented membrane models to generate line scans across membranes to calculate the distance between headgroup density peaks as a readout of membrane thickness [26–28]. Although individual line scans are noisy, averaging density profiles across entire membrane surfaces enables statistically robust detection of subtle (~ 2 Å) shifts in membrane thickness distributions. These analyses revealed that organellar membranes exhibit significant differences in global membrane thickness, with the OMM showing a ~0.4–0.6 nm reduction in thickness relative to the IMM, and up to >1 nm differences from other organelles such as the ER, plasma membrane, and nucleus, across mouse, human, *Saccharomyces cerevisiae*, and *Chlamydomonas reinhardtii* cells [27,28]. Membrane thickness can also vary locally within the same organelle membrane, as distinct IMM subcompartments exhibit significant differences (Figure 2d). This feature is not universal, however, since the rough and smooth ER membranes show comparable thickness (~3.7 nm) despite differences in protein association and function [27].

Some of the biophysical properties measured in cellular cryo-ET differ from those expected *in vitro* reconstituted systems. For example, we found that IMM thickness correlates positively with membrane curvature in cells [27], the opposite of the results observed *in vitro* [29]. This difference highlights the importance of the native cellular environment, where protein assemblies and lipid composition jointly shape membrane biophysical properties. Consistent with this, in a recent preprint, we found that the local membrane environment surrounding the mitochondrial protein prohibitin is significantly thinner than that of neighboring regions, with differences on the order of 0.2 nm [30]. This thinning persists even when accounting for differences among IMM subcompartments, suggesting that prohibitins either preferentially localize to thinner regions or actively remodel the lipid bilayer to create a unique membrane microenvironment. Another recent study demonstrated that a small population of the eukaryotic vault protein localizes to the endoplasmic reticulum (ER) membrane, where it associates with regions ~0.6 nm thinner than neighboring regions [31]. Together, these studies establish membrane thickness as a defining feature of local organelle membranes and a framework for testing how these biophysical properties influence protein and organelle function.

Protein patterning optimizes organelle biochemistry

Although contrast from the phospholipid bilayer can (at present) limit the direct visualization of smaller multipass transmembrane proteins in cellular tomograms, larger macromolecules with membrane-exposed domains can often be readily localized and mapped throughout organelle membranes. Significant advances in this area have been driven by studies of photosynthetic membranes in *Chlamydomonas reinhardtii*, where the high cytoplasmic water content enables clear visualization of membrane-embedded complexes in cryo-FIB-milled cells [11,32,33]. When combined with contrast-enhancing hardware, other systems, such as the membranes of cyanobacterial thylakoids [34] and the mouse rod outer segment [35], also exhibit clear

detection of membrane-embedded proteins. Another key advance came from software that reformatted membrane surfaces into flattened representations, allowing membrane-associated densities to be visualized and analyzed visually [33,36] and automatically [17,37] in two dimensions while preserving their native spatial context (Figure 2e).

These approaches have revealed that thylakoid membranes are not compositionally uniform but instead are subdivided into discrete regions, with photosystem I (PSI) and photosystem II (PSII) sharply segregated into non-appressed and appressed regions, respectively [33]. This patterning creates specialized membrane domains thought to help coordinate and tune photosynthetic reactions in cells. A recent study of thylakoid membranes in spinach shows that, despite drastic differences in overall membrane geometry, the molecular organization of PSI and PSII is strikingly similar to that in *Chlamydomonas reinhardtii*, with a sharp segregation of these complexes into appressed and non-appressed regions [38] (Figure 2e). This underscores how similar 'rules' for protein patterning can govern organelle membranes, even when they exhibit distinct geometries.

Mitochondrial redox complexes (i.e., respirasomes) do not exhibit the same degree of strict partitioning observed for photosystems, and instead appear more randomly distributed throughout cristae membranes [39]. Notably, a recent study identified a subpopulation of respirasomes localized to the IBM, the primary site of mitochondrial protein import. This observation raises the intriguing possibility that the observed patterning establishes local sites of coordination between the import and assembly of nuclear- and mitochondrial DNA-encoded subunits of respirasomes prior to redistribution within the cristae. The mitochondrial prohibitin complexes appear to exhibit differential enrichment across IMM subcompartments in different cell culture model systems [23,30,40]; however, whether this spatial patterning translates to unique functional consequences has not yet been determined.

Molecular sociology creates local membrane platforms for cellular processes

Many processes central to organelle physiology depend not only on internal machinery but also on coordinated interactions with the surrounding microenvironment. The conceptual roots of this phenomenon trace back to Palade's early electron micrographs of the rough ER, and cryo-ET has since extended these insights by mapping ribosomes engaged in distinct translational states to localized ER membrane regions [41].

Cryo-ET has revealed that ribosome-membrane sociology also defines local environments at the mitochondria. We identified a rare population of cytoplasmic ribosomes oriented for co-translational import at the outer mitochondrial membrane in *Saccharomyces cerevisiae* cells [42]. These ribosomes establish multiple physical contacts with the outer membrane and cluster into higher-order assemblies reminiscent of polysomes. Local membrane patch-based analysis showed that OMM-IMM spacing is reduced to ~10 nm locally beneath these ribosome clusters (Figure 2f), consistent with engagement with the mitochondrial import machinery [42]. In contrast, under glucose deprivation, cryo-ET revealed that translationally inactive ribosomes associate in ordered oligomeric arrays on the mitochondrial surface in a distinct orientation, tethered through the ribosomal protein Cpc2/Rack1 in *Schizosaccharomyces pombe* cells [43] (Figure 2g). These studies show that the OMM can host distinct ribosome-defined environments that modulate ribosome translation function.

Macromolecular sociology also plays a central role in defining organelle communication by forming membrane contact sites. In autophagy, the ends of the phagophore contact the ER, and cryo-ET has revealed that different textures of connecting densities, from ~20 nm rod-like densities to amorphous clusters, bridge these two membranes and define structurally distinct platforms for phagophore formation and expansion [22,23].

Another well-established contact site exists between the ER and the mitochondria, which support diverse functions, including lipid exchange, ion homeostasis, and mitochondrial dynamics. However, how functional specialization at these contact sites arises remains an exciting, ongoing area of investigation. Recent cryo-ET studies now suggest that distinct functions may emerge from the unique, local assembly of macromolecules at contact sites (Figure 2h). In yeast, the ER-mitochondria encounter structure (ERMES), known for lipid regulation, consists of heterogeneous clusters of bridge-like assemblies that tether membranes while still allowing flexibility in the inter-organelle spacing [44]. In mammalian cells, overexpression of the tethering protein FKBP8 reduces the distance between the ER and mitochondria, suggesting that the bridge formed between PDZD8 and FKBP8 proteins at the contact site may be more homogeneous in length [45]. In contacts associated with mitochondrial division, we and others have shown that constricted membranes are enriched with long, parallel bundles of cytoskeletal filaments [46] and granules containing the RNA-binding protein FMRP, which form ribosome-rich assemblies (Figure 2i) [47]. These studies illustrate the potential of cryo-ET to structurally decode the molecular logic of organelle contact-site physiology.

Future perspectives

Recent studies make it clear that cryo-ET can now provide systematic, quantitative structural characterization

of organelle membranes. However, translating these rich datasets into mechanistic and functional insights remains an ongoing challenge. The inherently static nature of cryo-ET snapshots makes it difficult to establish causality, such as distinguishing whether observed changes in organelle membrane geometry drive functional consequences or are coincidental features of a particular cellular state. Without such connections, some analyses risk remaining a descriptive, "stamp collection" of structures and measurements, rather than providing mechanistic insights. Integrating cryo-ET workflows with established cell biology tools, such as fluorescent functional readouts and genetic/pharmacological perturbations, will be key to molecularly dissecting how different local membrane environments form and which of these environments are functionally important. Beyond fundamental mechanistic insight, cryo-ET has also been used to reveal membrane remodeling in disease models, linking structural changes to pathological states [48–50]. Exciting advances in technologies that enable cryo-ET of patient-derived tissues have the potential to define the "ultrastructural signatures" that are diagnostic of or mechanistically contribute to disease pathogenesis [51].

A related challenge is that the selection of features to analyze in cryo-ET data remains largely user-defined, potentially limiting the discovery of new structural relationships or patterns that may be functionally important. Large-scale clustering analyses of ultrastructural features, such as those already available for membrane proteins [52], offer a path toward unbiased phenotypic classification of local membrane environments. As datasets and their corresponding quantitative measurements continue to accumulate in both individual labs and across public repositories [53], identifying which membrane phenotypes are most significant across different experimental conditions will become increasingly challenging for any individual investigator. The transcriptomics and proteomics fields have addressed these "big data" challenges across high-dimensional measurements and samples using AI-driven, unbiased classification to reveal new gene or protein expression patterns [54]. A similar opportunity exists for the cryo-ET field by applying these strategies to let the data, rather than our assumptions, define new membrane microdomains across experimental groups. By incorporating experimental metadata—such as treatment conditions, correlative fluorescence markers, and functional reporters of organelle activity—alongside cryo-ET datasets, these AI-assisted approaches may one day connect membrane structural changes to functional outputs and help identify which states truly matter to organellar physiology. Integrating these outputs with molecular dynamics simulations could move the field beyond static snapshots, toward predictive models of dynamic organelle states.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the author used Grammarly, Claude (Anthropic), and ChatGPT 5.2 in order to refine language, improve clarity and flow, and organize overarching concepts and ideas. After using this tool/service, the author reviewed and edited the content as needed and takes full responsibility for the content of the published article.

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