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Single-particle cryogenic electron microscopy structure determination for membrane proteins

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Membrane proteins are crucial to many cellular functions but are notoriously difficult for structural studies due to their instability outside their natural environment and their amphipathic nature with dual hydrophobic and hydrophilic regions. Single-particle cryogenic electron microscopy (cryo-EM) has emerged as a transformative approach, providing near–atomic-resolution structures without the need for crystallization. This review discusses advancements in cryo-EM, emphasizing membrane sample preparation and data processing techniques. It explores innovations in capturing membrane protein structures within native environments, analyzing their dynamics, binding partner interactions, lipid associations, and responses to electrochemical gradients. These developments continue to enhance our understanding of these vital biomolecules, advancing the contributions of structural biology for basic and translational biomedicine.

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Introduction

Membrane proteins play a pivotal role in all cellular processes, including signal transduction, transport mechanisms, cell communication, and others. They constitute approximately 30% of the human proteome and are the targets of over 60% of all therapeutic drugs [1]. For many years, despite intense efforts, progress in elucidating the high-resolution structures of membrane proteins was slow relative to soluble proteins. This challenge was mainly due to their inherent instability and amphipathic nature, which complicates their biochemical purification and crystallization. Traditionally, X-ray crystallography and nuclear magnetic resonance spectroscopy have been the primary techniques for structural determination, but these methods have substantial limitations with membrane proteins, particularly in their requirement for large amounts of stable and homogeneous samples.

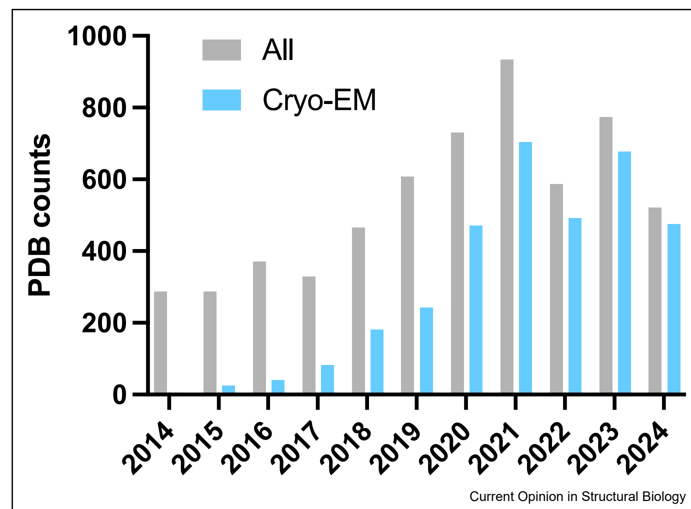
Cryogenic electron microscopy (cryo-EM) single-particle analysis (SPA) has revolutionized structural biology in the recent years, providing a powerful alternative for the study of membrane proteins at near-atomic-resolutions [2,3]. This technique allows for the visualization of proteins in near-native states without the need for crystallization, overcoming many of the barriers associated with other structural methods. The development of advanced direct electron detectors [4,5] and sophisticated image processing algorithms has significantly improved the resolution achievable by cryo-EM, making it possible to resolve structures of large, dynamic, and flexible membrane proteins at near-atomic resolutions. In 2014, only 1% of membrane protein structures deposited in Protein Data Bank were solved by cryo-EM. This number drastically increased to higher than 80% in the past couple of years (Figure 1).

In this review, we present current strategies and advancements in cryo-EM SPA for membrane protein structural studies. We discuss the challenges and solutions in single-particle sample preparation, highlight the latest developments in data processing, and examine the future directions of this rapidly evolving field. By integrating recent technological and methodological innovations, cryo-EM continues to push the boundaries of membrane protein research, offering unprecedented insights into their structure and function.

The choice of membrane mimetics

Purified membrane proteins require stabilization and solubilization in membrane mimetic systems that mimic the properties of biological membranes. Several membrane mimetics are used in structural studies, including detergents, amphipols, nanodiscs, and liposomes, as exemplified in Figure 2. Among these, detergents are

Figure 1



Number of membrane protein structures released in PDB each year between 2014 and 2024.
Data extracted from PDB, using the MemProtMD database (https://memprotmd.bioch.ox.ac.uk/) to identify membrane proteins. We direct interested readers to a comprehensive review article [6], which summarizes the membrane mimetics used to solve cryo-EM structures of membrane proteins between 2021 and 2022.
cryo-EM, cryogenic electron microscopy; PDB, Protein Data Bank.

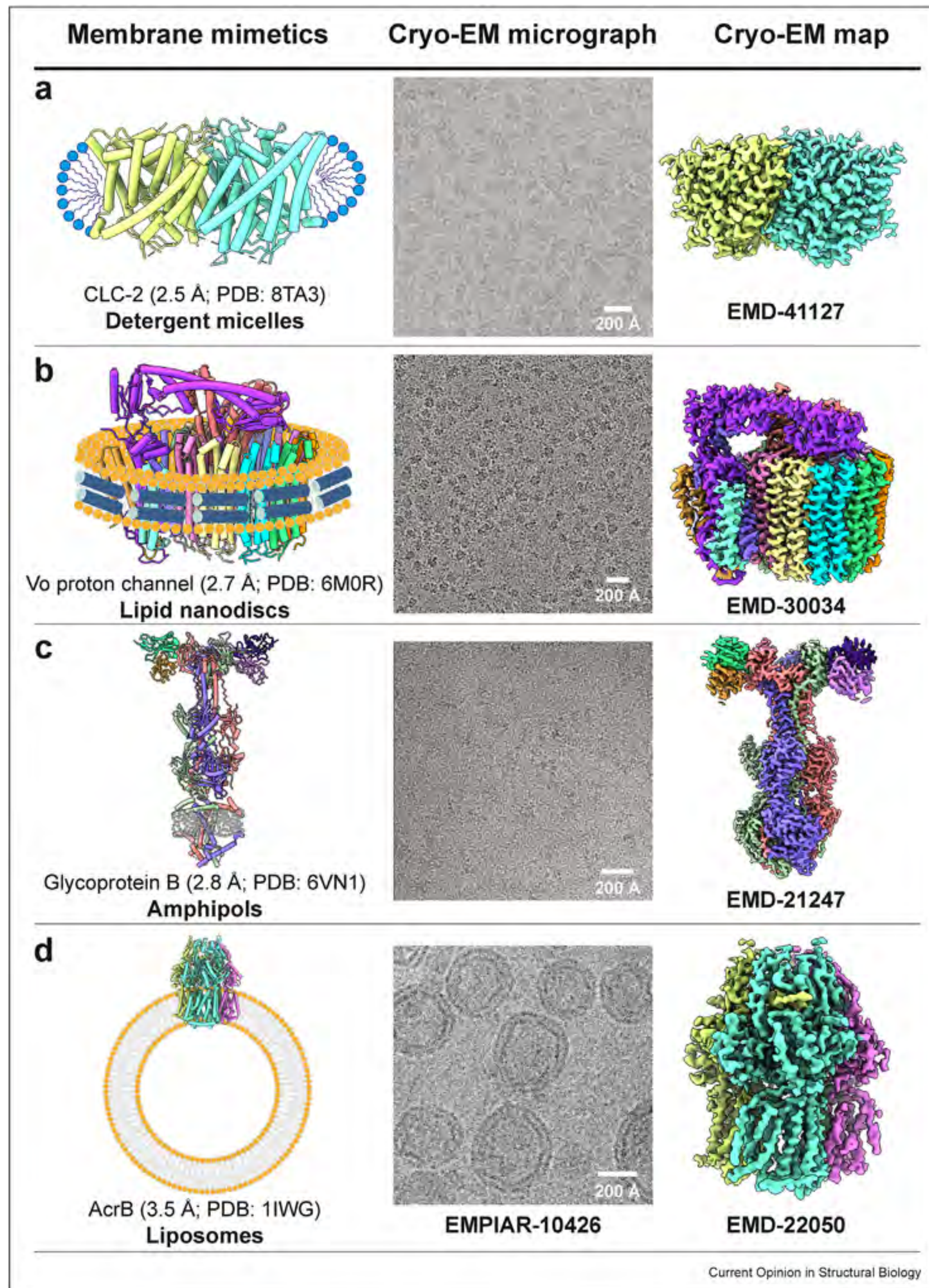
the most popular choice due to their simplicity and frequent use in protein extraction. Detergents with lower critical micelle concentrations (CMCs) or with minimal background in thin vitreous ice, such as n-dodecyl-β-D-maltoside, lauryl maltose neopentyl glycol, digitonin, and glyco-diosgenin, are particularly favored for cryo-EM SPA [6]. Their low CMC allows for lower concentrations in the sample, minimizing unwanted empty micelles and free detergent molecules that could reduce the signal-to-noise ratio in micrographs. It is worth noting that the choice of detergents is highly dependent on the protein of interest and typically requires empirical screening. Many proteins denature or aggregate during purification steps if a suboptimal detergent is used.

Although detergents have facilitated the determination of numerous membrane protein structures, they can sometimes disrupt the native structure of membrane proteins, leading to instability and aggregation. More critically, they may preserve stability but introduce structural distortions [7–9]. To mitigate these problems, solubilized membrane proteins can be reconstituted into detergent-free membrane mimetics [10]. Amphipols [11], amphiphilic polymers that wrap around membrane proteins to shield their membrane-interacting regions from water, have been successfully used in cryo-EM structure determination [12,13]. Lipid-based nanodiscs offer a more close-to-native approach. Nanodiscs consist of a small lipid bilayer patch encircled by scaffold proteins or polymers. Various types of nanodiscs with different scaffolds, including membrane-scaffold protein nanodiscs [14], peptidiscs [15], and Salipro [16,17], have been widely applied in cryo-EM SPA studies [18–20]. Despite many successful examples, these nondetergent membrane mimetics often require extensive optimizations of experimental protocol for a specific protein, without which many proteins become unstable and aggregate.

A recent approach reconstitutes membrane proteins into small, homogeneous liposomes for cryo-EM structure determination. Although this method presents challenges in particle picking due to strong lipid bilayer densities, high-resolution reconstructions can still be achieved through iterative particle picking and careful particle curation. This technique enables the exploration of different electrochemical environments across the membrane and the effects of membrane curvature on mechanosensitive ion channels [21–25]. One example is the 175 kDa dimeric OSCA channel structure, resolved at a resolution better than 4 Å [26].

A promising new strategy involves a complete elimination in the use of detergent in order to preserve native interacting lipids and cofactors. Methods for detergent-free direct extraction of proteins from cellular membranes include using styrene maleic acid lipid particles [27], cycloalkane-modified amphipols [28], or Salipro [29]. An exciting alternative involves preparing proteoliposomes directly from cells without the need for additional scaffold proteins or polymers [30,31]. This

Figure 2



Examples of membrane proteins reconstituted in various membrane mimetic systems used in cryo-EM studies at near atomic resolutions. **(a)** Chloride channel CLC-2 in detergent LMNG [76]. **(b)** Vo proton channel in lipid nanodiscs [69]. **(c)** Glycoprotein B in amphipol [12]. **(d)** AcrB in liposomes [21]. For each example, the following are shown: a schematic representation of the membrane mimetic, a representative cryo-EM micrograph, and a cryo-EM map. Additional examples of cryo-EM structures for each membrane mimetics are provided here. Detergents: 7KTX/EMD-23033, 6VX3/EMD-21428; Nanodiscs: 6UZH/EMD-20959, 7T0W/EMD-25583; Amphipols: 7QHA/EMD-13968, 6WU3/EMD-21904; Liposomes: 7WLT/EMD-32592, 8EOW/EMD-28487.
cryo-EM, cryogenic electron microscopy; LMNG, lauryl maltose neopentyl glycol.

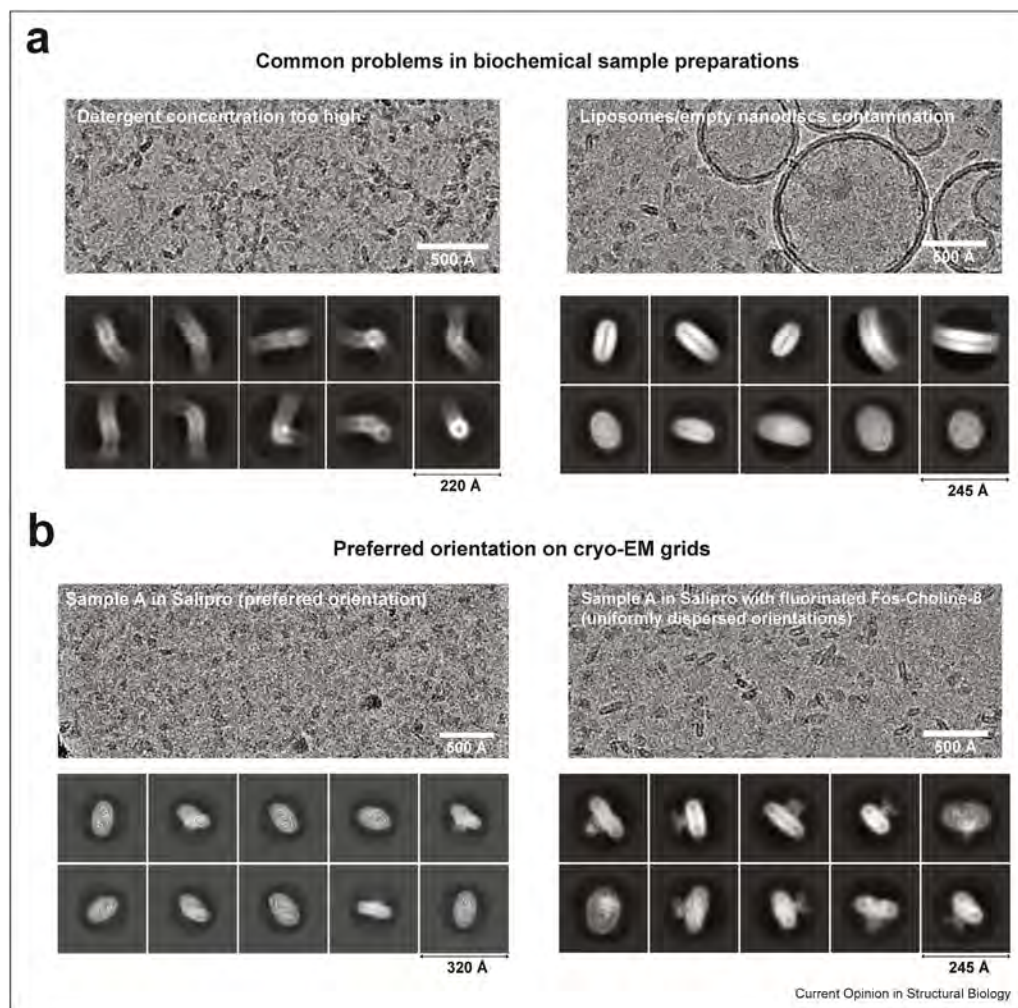
approach, in theory, is generalizable to a wide range of membrane proteins.

Common problems in membrane protein samples for cryo-EM

Despite the success of the various approaches mentioned, sample preparation for cryo-EM SPA of a new membrane protein remains a significant bottleneck in structure determination [32]. One of the primary challenges arises from the amphipathic molecules used to stabilize membrane proteins. For instance, excess detergent can lead to large quantities of empty micelle particles, which increase background noise and hinder data quality (Figure 3a). Similarly, when using lipid nanodiscs, the presence of empty nanodiscs or liposomes can complicate data processing, ultimately obstructing high-resolution reconstruction (Figure 3a). Extensive data processing could be applied to handle these datasets, but a more homogeneous sample is preferred to expedite the structural determination.

In addition to these challenges, membrane proteins, like soluble proteins, are often prone to preferred orientation at the air—water interface. Common strategies to mitigate this issue include tilt data collection [33], the use of ultrathin (2—3 nm) carbon [34], graphene [35] or graphene oxide grids [36], and the addition of detergents. However, these approaches have their challenges and disadvantages such as lower data acquisition efficiency, increased background noise, and production difficulties. It has been reported, both in the literature and in our own experience, that using fluorinated

Figure 3



Common problems in membrane protein cryo-EM dataset.
(a) Left panel: a sample with too high concentration of detergent LMNG shows fiber-looking micelles. Right panel: a sample with empty nanodiscs and liposomes. **(b)** Left panel: A membrane protein in Salipro showed mostly the top view in the 2D classification. Right panel: Addition of 0.5 mM of fluorinated Fos-Choline-8 improves the particle orientation distribution.
cryo-EM, cryogenic electron microscopy; LMNG, lauryl maltose neopentyl glycol; PDB, Protein Data Bank.

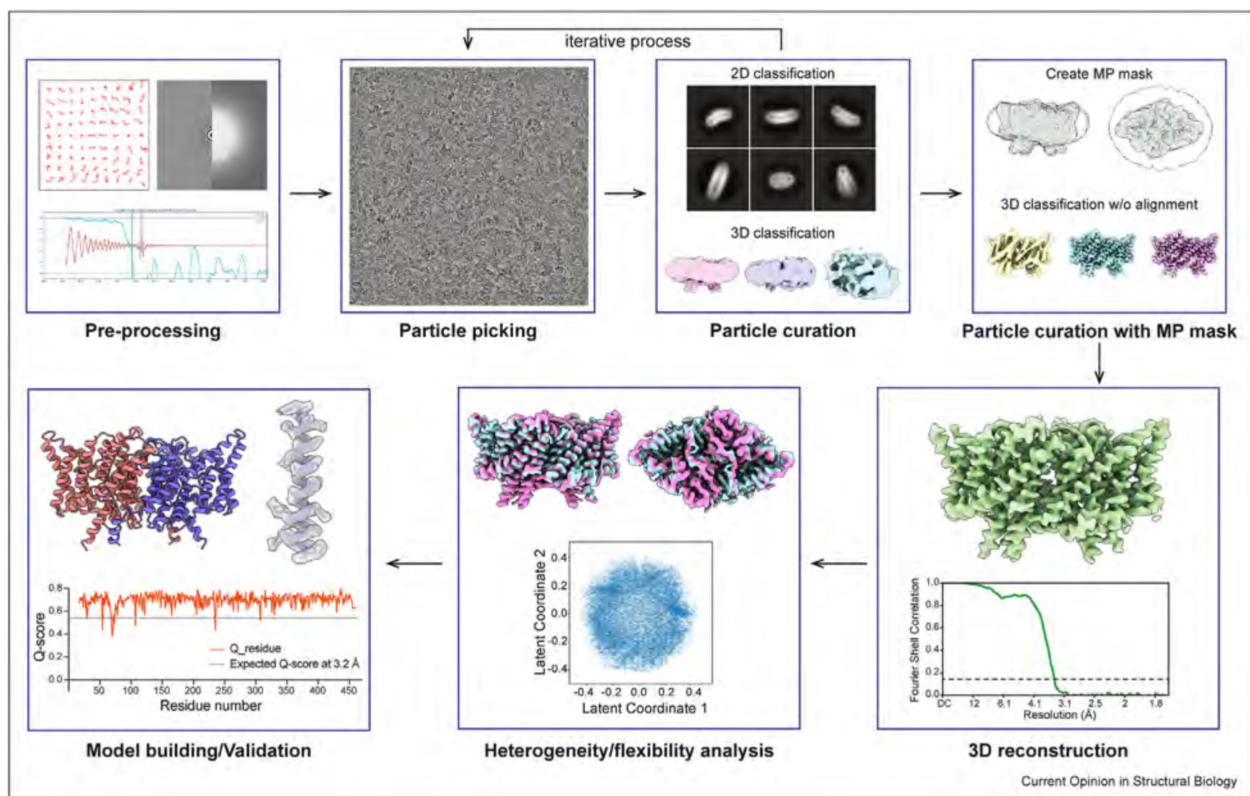
detergents, such as fluorinated fos-choline-8, can effectively address the issue of preferred orientation without compromising membrane protein integrity [19,37] (Figure 3b).

Advancement in SPA data analysis

The SPA data processing workflow for membrane proteins closely resembles that used for soluble proteins (Figure 4). The most commonly used software programs are Relion [38] and CryoSPARC [39]. The overall workflow involves preprocessing (motion correction and contrast transfer function estimation), particle picking, particle curation through 2D and 3D classifications, and final 3D reconstruction. It is worth noting that particle picking is often challenging with the low signal-to-noise ratio of cryo-EM data. Therefore, it is a common practice to iteratively refine particle picking by alternating between picking and curation to achieve more accurate results.

A distinctive challenge for membrane protein cryo-EM data is the strong density contributions from the membrane mimetics. These mimetic densities may dominate the low-frequency signal, which helps the alignments of low-resolution features but obscures the alignment of high-frequency protein structural details. To address this challenge, focused classification or refinement steps are often used, using masks to exclude membrane mimetic densities. This process not only improves alignment but also enables an additional curation step to exclude empty micelles, nanodiscs, or poorly aligned particles. In addition, nonuniform refinement in cryoSPARC [39] that adopts per-region anisotropy weighting significantly reduces the contribution of high-frequency signal from the membrane

Figure 4



Standard Cryo-EM data processing workflow as exemplified by cryo-EM studies of a chloride transporter (CLC-ec1) at 3.2 Å (unpublished). Raw movies go through preprocessing including motion correction and contrast transfer function estimation. The first particle picking is done by blob picking followed by particle curation using 2D and 3D classifications. The good particles can be used as templates or training sets for machine learning–based particle pickers to improve particle picking. Specific to membrane proteins, using a mask to perform focused classification on the protein often improves the result by removing the undesired signal from membrane mimetics. The final good particles are used for the 3D refinement and further heterogeneity/flexibility analysis. A final cryo-EM map with clear backbone and side-chain densities, usually with a resolution better than 4 Å, would be used for model building, with Q-scores at all amino acid residues to ensure robust validation.
cryo-EM, cryogenic electron microscopy.

mimetics, often leading to higher-quality maps. After this, heterogeneity and flexibility analysis can be applied to explore compositional and conformational variations, which is important for understanding the protein's biological functions. The features of the membrane proteins in the final map can often be further improved by local anisotropy sharpening in Phenix [40].

Once a high-quality cryo-EM map is generated, molecular models can be built using tools such as Phenix [40], Refmac [41], or the more recent ISOLDE [42]. Validation tools, such as Q-score [43] and MolProbity [44], help ensure that the models are chemically accurate and the map-model fit is optimal.

Recent advancements in machine learning have dramatically enhanced several stages of the cryo-EM data processing pipeline. Tools like Topaz [45], DeepPicker [46], and crYOLO [47] have improved the accuracy and efficiency of particle picking. Machine learning algorithms have also been applied to enhance 3D reconstructions and refinements, with notable tools including blush [48], DRGN-AI [49], and GMM [50]. Additional progress has been made in heterogeneity and flexibility analysis through methods like CryoDRGN [51], e2gmm [52], 3DFlex [53], 3DVA [54], Zernike3D [55], and DynaMight [56]. Machine learning–based tools for map manipulation, such as spIsoNet [57] and DeepEMhancer [58], have also contributed to improving the quality of maps, while automated model building has been revolutionized by algorithms like ModelAngelo [59]. When prior structural information is lacking, tools like AlphaFold [60,61] that predict protein structure with sufficient accuracy may be used to generate an initial model, followed by further refinement.

In situ single-particle analysis

The emerging field of *in situ* SPA marks a new era in structural biology by enabling visualization of proteins within the native cellular environments or in the context of an intact physiological setting. It has been successfully applied to soluble proteins, such as ribosomes and other large cellular complexes in cells without biochemical purification. However, its application to membrane proteins remains challenging due to their heterogeneity, low abundance, and the ensuing difficulties associated with particle picking and alignment. The feasibility of membrane protein *in situ* SPA was first demonstrated using viral spike proteins on intact and infectious virions, benefiting from the relatively small size of the virions, as well as the abundance and relative homogeneity of the membrane protein with transmembrane and soluble domains [62–64].

Recently, several studies determined high-resolution membrane protein structures in more challenging physiological contexts, directly from organelles or cells. These studies revealed unprecedented insights into membrane-protein behavior in native environments. Two studies reporting structures of the proton pumping vesicular-type ATPase (V-ATPase) in isolated native synaptic vesicles identified bound protein synaptophysin that was not observed in the previous structural studies of purified V-ATPases [65,66]. High-resolution structures of mammalian respiratory supercomplexes obtained directly from porcine mitochondria revealed different conformations of the complexes along with their interactions with surrounding lipids [67]. Strikingly, in a recent study at the plasma-membrane level, a near–atomic-resolution map of a red algal light-harvesting complex from *Porphyridium purpureum* was resolved [68]. To achieve this feat, focused ion beam milling was used to create thin lamellae of the algal cells, allowing for detailed cryo-EM analysis. Together, these studies highlight the broad potential of *in situ* cryo-EM SPA to understand membrane proteins in their native environments.

What can be learned from cryo-EM structures of membrane proteins?

With ongoing advancements in both hardware and software, cryo-EM SPA will continue to be an indispensable tool for addressing increasingly complex biological questions. Here, we summarize five key focus areas where cryo-EM SPA experiments are swiftly yielding new insights into membrane protein structure and physiology. We anticipate that these areas will be primary directions for the future.

- **Visualization of water and ligands**

Cryo-EM structures with resolutions approaching *2* Å offer a wealth of detailed information undetected in lower-resolution images [20,69]. Detection of ordered waters, ions, and cofactors will enable chemical mechanistic studies that are not feasible without detailed structural knowledge of these critical components. For instance, the structures of an organic anion transporter with ∼2.5-Å resolution revealed ordered water molecules, bound substrates, and lipids that are essential for understanding its functions [70]. High-resolution structures not only advance our understanding of biological mechanisms but also enhance the drug development field. The detailed binding information revealed in the structures will directly inform the optimization of therapeutic compounds, facilitating effective and targeted treatments [71].

- **Protein dynamics**

Cryo-EM SPA captures multiple conformational states of proteins in the vitreous ice, offering an opportunity to

understand the protein dynamics that occur in the aqueous environment at physiological temperatures. As mentioned earlier, many sophisticated machine learning–based tools have been developed to extract this information. In one example, using the 3DVA algorithm [54], the temporal order of events driving G-protein activation in response to GTP binding was revealed in high-resolution detail [72]. This study demonstrates the potential of the cryo-EM SPA, in combination with other biophysical techniques and MD simulation, for a deeper understanding of dynamic events that underlie complex physiological processes.

- **The effects of lipid environments**

The effect of lipid environments, including lipid compositions and the lipid bilayer's physicochemical properties, on membrane protein structures are still poorly understood. Cryo-EM SPA facilitates systematic studies of membrane protein structures in various lipid environments. Recent studies, for example, have revealed different conformations of ligand-gated ion channels [73] and nicotinic acetylcholine receptors [74] in distinct lipid environments. In addition, contrast-enhancing brominated lipids were used to accurately identify the bound lipid in the TRPV1 binding pocket [75]. We expect investigations will continue to reveal new ways in which membrane properties influence the structures of embedded proteins.

- **The effects of electrochemical gradients**

Membrane proteins inhabit asymmetric environments where transmembrane electrochemical gradients are continuously generated and maintained. The emerging liposome-based approach described earlier holds great promise for understanding how these gradients influence structure and function. For example, cryo-EM structures of a voltage-dependent K^+ channel embedded in liposomes with a transmembrane voltage imposed offer an unprecedented view of how these channels respond to transmembrane voltage, the primary physiological stimulus driving their function [23]. The liposome approach opens myriad possibilities to investigate protein conformational changes under transmembrane gradients relevant to each protein's physiology, a capability that is inaccessible with membrane mimetics.

- **Native structure determinations**

Obtaining native structures of membrane proteins in their original lipid environment provides more insights into their functions and mechanisms. Detergent-free membrane protein extraction enables investigations of native lipids, interacting cofactors, and ligands. It also simplifies the protein purification process and still provides suitable samples for cryo-EM SPA with little background.

Another exciting frontier is *in situ* SPA. This method examines proteins within their native membranes, allowing for unperturbed interactions with endogenous lipids and binding partners. As a result, it sets a benchmark for accurately representing the interactions, conformational states, and dynamics of biomolecules as they function naturally. Although *in situ* SPA is currently limited to abundant protein with large molecular weight for easier identifications, we anticipate that more *in situ* structural studies of smaller targets will emerge with the development of the technique.

Declaration of competing interest

The authors declare no conflict of interest that appears to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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