Title

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Membraneprotein structural biology in the era of single particle cryo-EM

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Abstract

In the past few years, significant technological breakthroughs in single particle cryo-electron microscopy enabled a “resolution revolution” of this technique. It also changed structural biology in an unprecedented way. For many biological macromolecules, obtaining well-ordered crystals of suitable size is no longer a prerequisite for determining their atomic structures. One of the most impacted areas is the structural biology of integral membrane proteins. New structures are now determined at a rapid pace. Despite these advances, further technological developments are still required to overcome new technical challenges that face membrane protein structural biology. In this review, I attempt to discuss some of these challenges.

Introduction

Structure determination of integral membrane proteins is one of the greatest challenges in structural biology. In the 1970s, electron crystallography was developed as a promising method for determining the structure of membrane proteins in their native lipid environment [1]. This method combines both imaging and diffraction techniques to produce atomic structures of membrane proteins [2–4]. Because the method requires obtaining first well-ordered two-dimensional (2D) crystals, however, its output has been limited to fewer than a handful of unique atomic structures determined to date [5,6]. the predominate technique in membrane protein structural biology had been X-ray crystallography — indeed, most of the membrane protein structures in the protein databank were determined thus [7]. While the technology of X-ray crystallography itself is well-established, and in most cases the throughput from data acquisition to structure determination is extremely efficient, crystallization remains a major bottleneck for challenging membrane proteins, particularly for mammalian membrane proteins [8,9].

Unlike crystallographic approaches, single particle cryo-electron microscopy (cryo-EM) determines structures of biological macromolecules by averaging electron microscopy

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images of target molecules embedded in a thin layer of vitreous ice in random orientations [10]. Because it does not require either crystallization or absolute sample homogeneity, it has been used to study large integral membrane proteins that are obviously refractory to crystallization, such as the calcium release channel ryanodine receptor [11]. For a long time, however, the resolutions of most membrane protein structures obtained by this method were at the level of "blobology" at best. The structural analysis of various transient receptor potential (TRP) channels is one example [12–14]. In a few isolated cases, structures were produced that have turned out to be incorrect [15,16]. For many years, scientists invested great time and effort to improve every aspect of single particle cryo-EM so that such mistakes could be avoided and reliable structures could be produced and validated [17,18]. Consequentially, the resolutions of single particle cryo-EM structures of some large integral membrane proteins improved considerably [19].

Recent technological breakthroughs have since brought forth a "revolution" in cryo-EM [20]. among the most notable is the introduction of the direct electron detection camera [21] and related techniques of correcting beam-induced image motions [22–25], as well as various novel image processing algorithms [26,27]. This resolution revolution has transformed single particle cryo-EM from a method complementary to X-ray crystallography to a method dominating atomic structure determinations in many areas of structural biology [20]. Membrane protein structure determination is one the first few areas in structural biology that has been greatly impacted by the revolutionary technological breakthroughs in single particle cryo-EM.

Membrane protein structure determination at a rapid pace

Prior to 2014, all deposited cryo-EM PDB coordinates of membrane proteins were from electron crystallography. Since the first atomic structure of a membrane protein was determined by single particle cryo-EM in 2013 [28,29], the number of membrane protein structures determined every year using this method increases by leaps and bounds. In 2014, less than 5% of total membrane protein structures determined in that year were by cryo-EM (including both single particle cryo-EM and electron crystallography). This number jumped to about 27% in 2017 and grew to more than 35% by the first few months of 2018 (Figure 1). A closer look at some specific membrane protein families shows that the contribution of single particle cryo-EM is much more impressive than even what these numbers imply. In some major families of integral membrane proteins, most new structures were determined by single particle cryo-EM, and being done so at a pace faster than ever before.

Take TRP channels as an example. It is a major ion channel superfamily, with seven subfamilies [30,31]. Significant efforts from many crystallographic laboratories around the world failed to produce crystal structures of any member of this superfamily, except a few small domains and pieces [32,33]. Since the atomic structure of TRPV1 was determined in 2013 [29], structures of all seven TRP channel subfamilies have now been determined by single particle cryo-EM [29,34–39]. In comparison, there are now two crystal structures from the TRPV subfamily, and both were determined at lower resolutions than the cryo-EM structures of the same proteins [40,41]. In some other major membrane protein families, such as pentameric ion channels [42], ABC transporters [43], and potassium channels [44],

most new structures are from cryo-EM. For some large integral membrane proteins and protein complexes, such as respiratory chain complex [45,46] and megacomplexes [47], mechanosensing piezo ion channels [48,49], and ATP synthases [50,51], all new structures are determined by single particle cryo-EM. Some of them are reviewed in this same issue.

Nowadays, most single particle cryo-EM data are acquired in an automated fashion [52,53] enabled by highly stable electron microscopes equipped with direct electron detection cameras and operated by experienced staff scientists, in a fashion somewhat similar as X-ray diffraction data is collected at a beamline. Together with robust and streamlined image processing pipelines [26,54,55], high-resolution structure determination by single particle cryo-EM has become easier and more accessible than ever before. Such technological advances clearly facilitate the atomic structures of membrane proteins being determined at a rapid pace.

Challenges in the new era of membrane protein structural biology

In the era of single-particle cryo-EM, it is necessary to be aware not only of what the modern cryo-EM technology can do, but also of some existing shortfalls of the technology, and new challenges and bottlenecks in the determination of membrane protein structures.A few of them are discussed in the following.

Identifying bound ligands with accuracy and precision

While the resolutions of many cryo-EM membrane protein structures are sufficient for the atomic model building based on primary sequence, the map quality is often insufficient to identify non-protein densities such as bound ligands or lipids accurately. In such cases for ligands, it becomes necessary to compare the density maps determined from samples with and without the ligand to identify the location of bound ligands [28], a strategy that can be even further validated by mutagenesis experiments. Lipids with characteristic shapes, such as phosphatidylinositol lipids [56,57] and cholesterol [34], are readily identifiable given maps of sufficient resolution. For most annular lipids, however, assigning specific lipid identity is somewhat arbitrary.

Identifying bound ions that play critical functional roles is more challenging. Calculating difference maps between structures with and without bound ions, checking the consistency in both half maps, and checking ion coordination by side chains from neighboring residues are all necessary procedures to confirm the identity of bound ions [34]. Mutagenesis experiments add more confidence to the assignment. Identifying metal ion densities located at symmetry axis, such as along the ion permeation pathway of a tetrameric ion channel, is even harder. Densities situated at any symmetry axis could be due either to metal ions coordinated by the ion permeation pathway or simply noise densities enhanced by symmetry [34]. Unlike X-ray crystallography, single particle cryo-EM has no method equivalent to anomalous diffraction to identify the locations of certain types of metal ions unambiguously. This ambiguity in the structure is likely to be present even when the resolution of cryo-EM density maps is improved to beyond 3 Å. In comparison, crystal structures of TRPV4 and TRPV6 show clear densities of ions in the permeation pathway, even at relatively lower resolution than the corresponding cryo-EM structures [40,41]. For cryo-EM, having similar

densities at the same location in both half maps or in multiple maps determined independently can provide some assurance that the density is not contributed by noise. Otherwise, one has to be very cautious in interpreting such density as bound ions.

Protein quality matters

While crystallization is no longer an absolute necessity for atomic structure determination, obtaining biochemically well-behaved proteins is still an essential requirement. One could certainly argue that the beauty of single particle cryo-EM lies in its ability to classify particle images of different conformations and that sample homogeneity is thus not a necessity. This is true—to an extent. In practice, samples that are biochemically stable and conformationally homogeneous often lead to high-quality structures quicker. Tools developed by crystallographers, such as fluorescent size exclusion chromatography [58], play a significant role in optimizing sample preparation. Negative stain EM can also be used to evaluate sample homogeneity [36]. In summary, with our current technology, the time-consuming processes of perfecting sample preparation and optimizing sophisticated image classification algorithms to compensate for non-homogeneous samples are still necessary to obtain high-resolution structures.

If the target proteins are simply not homogenous enough to produce a high-resolution structure, various image processing techniques, such as extensive classifications or focused alignment [59], may improve the resolution of local regions. If functionally critical regions of a protein are still not fully resolved, one may have to expend valuable time and effort optimizing the biochemical preparation of the proteins, whether by screening orthologues or optimizing protein expression and purification. There is often no high-throughput screening method to speed up this process, and one is instead left waiting helplessly until the end of image processing to know if the optimization efforts have improved the situation or not. Assuming one has sufficient access to high-end electron microscopes and computational power, obtaining biochemically well-behaved proteins is probably the most time-consuming step of the process. More efficient approaches are needed to overcome this bottleneck.

Protein size also matters

Integral membrane proteins being studied using single particle cryo-EM are either reconstituted into lipid nanoparticles [56,60] or solubilized in detergents or detergent-like amphipols [29]. Substantial amounts of unstructured mass from detergent micelles or lipid nanoparticles around the transmembrane domain contribute low-resolution signals but high-resolution noise, which inevitably influence or bias image alignment. Contrast enhancing approaches, such as using phase plate, have limited help in this regard, as the contrast of unstructured mass increases with the target proteins. For membrane proteins with large and ordered soluble domains to dominate accurate image alignment, the resolution is often influenced more by the level of conformational homogeneity than the accuracy of image alignment. For membrane proteins with small or no ordered soluble domains, it is harder to achieve accurate image alignment and thus high-resolution structure determination. Symmetry facilitates accurate image alignment and mitigates the need for substantially ordered soluble domains outside of the membrane. Most single particle cryo-EM membrane protein structures thus far have either some ordered soluble domain or symmetry.

Is it possible to determine atomic structures of small integral membrane proteins without symmetry or much of a soluble domain and being reconstituted in lipid nanoparticles? There is no clear answer to this question, but it is difficult with no doubt. good example is the family of G-protein coupled receptor (GPCR). Early breakthroughs made GPRCs feasible targets for X-ray crystallography [61], but most of them are still considered too small to be studied directly by single particle cryo-EM. The situation is different for GPCR/G protein complexes, which is becoming more feasible by cryo-EM, as demonstrated first by [62] and followed with many more cryo-EM structures of various GPCR complexes. It seems that adding some ordered soluble mass with recognizable structural features—for example, forming GPCR/G protein complexes [62] or binding a conformational specific Fab [63]—can provide a fiducial marker with which to facilitate image alignment of such small proteins and make it feasible for cryo-EM structure determination.

The broader impact of single particle cryo-EM to membrane protein structural biology

Nowadays, obtaining well-behaved proteins and getting access to the technology are the new limiting factors when determining atomic structures of many membrane protein targets that were out of reach in the past. The cryo-EM community's continuous training efforts are mitigating that second limiter, making the technology more accessible to the wider structural biology community than ever before. Thus, proteins with good expression yield and biochemical stability become new low-hanging fruits for structure determinations. While the structures of new membrane proteins are now being determined faster than ever, the competition is also becoming unprecedentedly tense. In many cases, different laboratories unknowingly focus their efforts on the same or similar targets. It is quite common now to see several structures of the same protein being published at the same time by multiple different laboratories around the world. Recent examples include TRPML [37,64–67], TRPM [34,68–70] (Figure 2), and Piezo [48,49,71].

While competition can, at times, spur progress, very intense competitive environment like that present within the cryo-EM community today could also impede productivity and stymie new discovery. Nowadays, it is rare to hear presentations of on-going and unpublished work, and some published results have visible signs of "being rushed" to get the results out. This winner-takes-all culture depreciates the value of some high-quality work, only because being submitted for publication a few weeks too late can significantly lessen the perceived impact of a publication. The constant worry of being scooped by unknown competitors could lead an atmosphere that discourages both risk-taking and, most importantly, communication. Collaboration and discussion are vital in all fields of modern research but is especially important in cryo-EM, which makes the practice of "defensive" science particularly ruinous for our field. While improving cryo-EM technology is important, changing the culture is also necessary. Releasing cryo-EM structural studies in BioRxiv such as seen in the recent release of TRPC channel structures [39,72–74] may set a good example in this direction.

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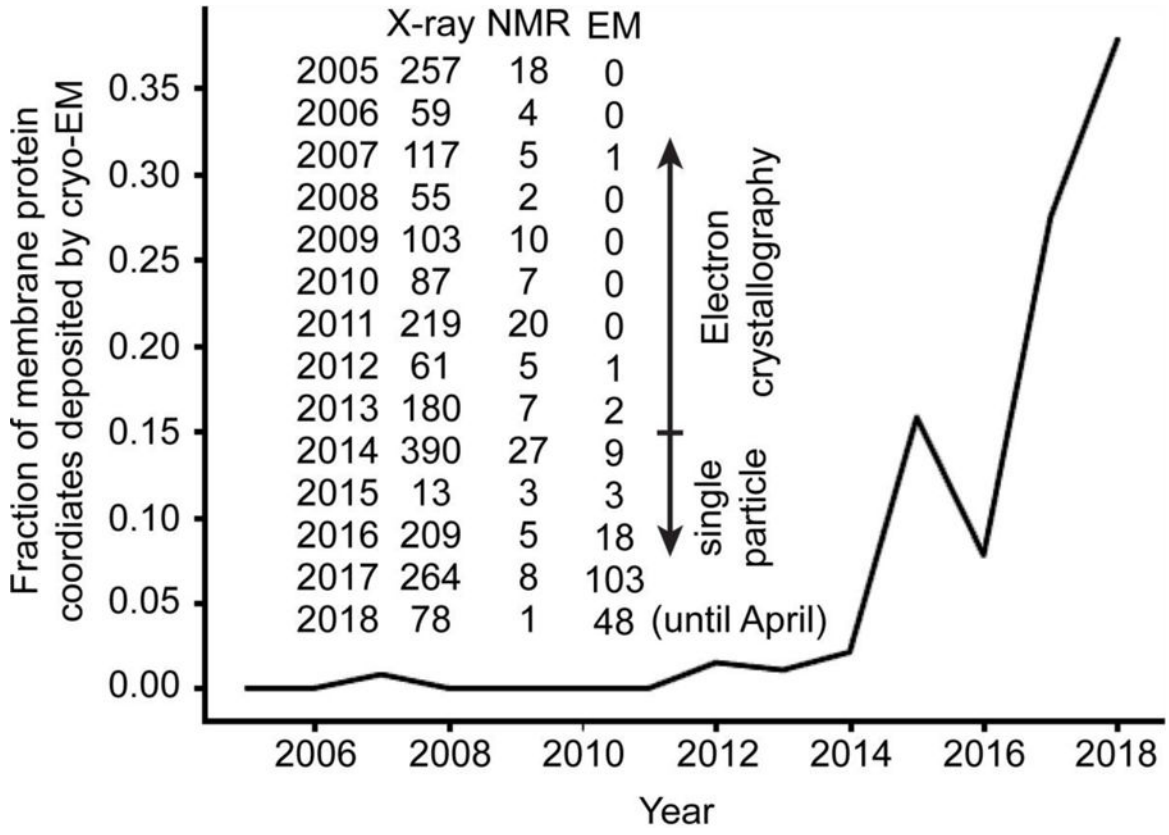

Figure 1. Increase of number of cryo-EM structure of membrane proteins

This graph shows the percentage of cryo-EM membrane protein structures in every year since 2005. The percentage increase to close to 40% in the first three months of 2018. It shows an increasing role of single particle cryo-EM in structural biology of membrane proteins.

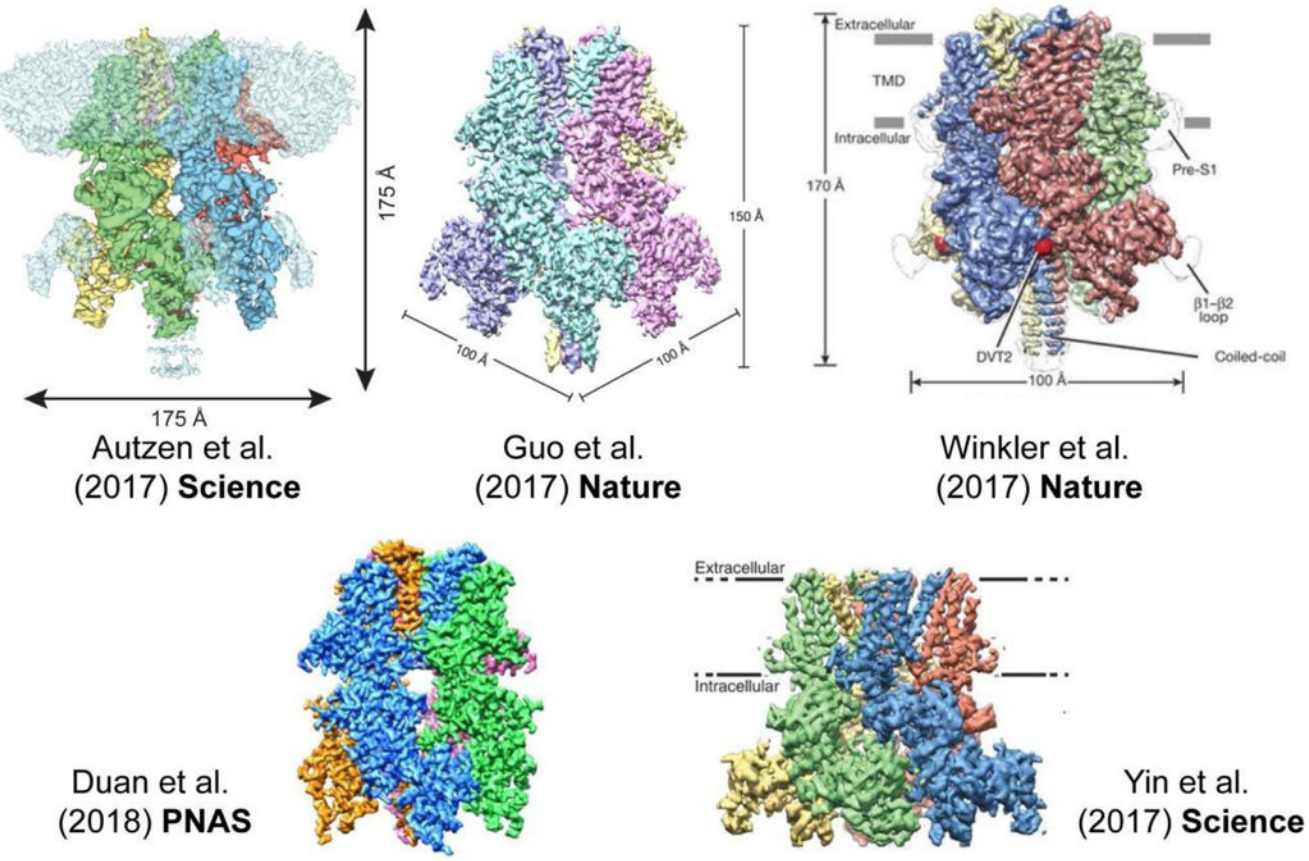

Figure 2. Cryo-EM structures of TRPM

In December 2017, three structures of TRPM4, including human TRPM4 with and without Ca2+ bound [34], mouse TRPM4 with and without ATP bound [69], human TRPM4 with decavanadate bound [70] and one structure of TRPM8 [75] were published in the same week. Another structure of human TRPM4 apo structure was [68] published two months later. Structure information of all these works are complementary with each other, provide substantial amount of structural information of this subfamily of TRP channel.