Cryo-EM of ABC transporters: an ice-cold solution to everything?

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High-resolution cryo-EM has revolutionized how we look at ABC transporters and membrane proteins in general. An ever-increasing number of software tools and faster processing now allow dissecting the molecular details of nanomachines at atomic precision. Considering the further benefits of significantly reduced sample demands and increased speed, cryo-EM will dominate the structure determination of membrane proteins in the near future without compromising on data quality or detail. Moreover, improved and new algorithms make it now possible to resolve the conformational spectrum of macromolecular machines under turnover conditions and to analyze heterogeneous samples at high resolution. The future of cryo-EM is, therefore, bright, and the growing number of imaging facilities and groups active in this field will amplify this trend even further. Nevertheless, expectations have to be managed, as cryo-EM alone cannot provide an ultimate answer to all scientific questions. In this review, we discuss the capabilities and limitations of cryo-EM together with possible solutions for studies of ABC transporters.

Keywords: ABC transporters; conformational spectrum; cryo-EM; membrane proteins

The resolution revolution [1] in cryo-EM marks a new chapter in structural biology, especially for membrane proteins [2,3]. Nevertheless, when the first subnanometer cryo-EM reconstruction of an ABC transporter appeared in 2015 [4], no one would have predicted that cryo-EM could soon rival with X-ray crystallography for ABC transporters at resolutions comparable to those of X-ray crystallography already emerged in 2016 [5,6] and, suddenly, cryo-EM-based drug design with ABC transporters appeared on the horizon [7]. It took only another three years to pass the 3 Å barrier for this class of transporters [8].

Owing to significant technological developments of hardware and software [9–15], cryo-EM is growing exponentially, and new breakthroughs in resolution

Abbreviations
ABC, ATP-binding cassette; ABCA1, ATP-binding cassette super-family A member 1; ABCB11, ATP-binding cassette super-family B member 11; ABCB4, ATP-binding cassette super-family B member 4; ABCD4, ATP-binding cassette super-family D member 4; ABCG2, ATP-binding cassette super-family G member 2; BCRP, breast cancer resistance protein; CFTR, cystic fibrosis transmembrane conductance regulator; CHS, cholesteryl hemisuccinate; cryo-EM, cryo-electron microscopy; cryo-ET, cryo-electron tomography; DDM, n-dodecyl-β-maltoside; DIBMA, diisobutylene/maleic acid; ECVs, extracellular vesicles; GDN, glyco-diosgenin; KATP, ATP-sensitive potassium channel; LMNG, lauryl maltose neopentyl glycol; MRP1, multidrug resistance-associated protein 1; NBD, nucleotide-binding domain; PCAT, peptidase-containing ATP-binding cassette transporter; Pgp, P-glycoprotein; PLC, MHC-I peptide loading complex; SMA, styrene/maleic acid; SUR1, sulfonylurea receptor 1; TAP, transporter associated with antigen processing; TMD, transmembrane domain; TmrAB, Thermus thermophilus multidrug resistance protein A and B; UDM, n-undecyl-β-maltoside.
and lowest analyzable molecule size are constantly witnessed [16–18]. The field is expanding not only by the amount of resolved structures, but also by the number of users and developers, as more and more researchers are turning to cryo-EM to answer their specific scientific questions. This popularity is certainly based on the fact that samples are imaged in vitrified suspension, which abolishes the need for crystallization, the major obstacle to X-ray structure determination of membrane proteins. As such, cryo-EM enabled the analysis of ABC transporters that defied crystallography, such as the cystic fibrosis transmembrane conductance regulator (CFTR) [6,19–23] and ABCG2 [7,24–26], also known as breast cancer resistance protein (BCRP). Moreover, the low sample amount requirements in cryo-EM provide the opportunity to study low-abundant, native, fragile, or flexible complexes [27–34], while the high tolerance to various hydrophobic environments [35–41] enables structure determination of ABC transporters not only in different detergents [5,6,28,29,42–52], but also within a lipid environment [8,24,53–56]. Additionally, cryo-EM can dissect conformational and compositional heterogeneity, which sets the ground to study structural dynamics and assembly states of ABC transporters and their complexes [8,28]. However, cryo-EM must not be seen as the silver bullet that can be directly used to resolve all controversies within the ABC-transporter field. In this review, we provide a personal perspective on the benefits and limitations of cryo-EM for structural studies of ABC transporters and highlight the importance of integrative approaches.

Cryo-EM and insights into ABC-transporter dynamics

One transporter – multiple structures

In cryo-EM, individual proteins are not confined by crystal packing, but suspended within a thin layer of aqueous solution, which is rapidly plunged into liquid ethane for vitrification [57]. This process is tolerant to minor modifications of the sample, as for example, addition of nucleotides or small alterations of the construct. Therefore, unlike in crystallography, many different structures of the same transporter, trapped in various conformations, can be prepared and analyzed in a timely manner, as demonstrated by the three seminal publications on CFTR within less than a year [6,19,20]. Improvements in microscope hardware and software, together with the larger computing capacities and live data processing, are continuously accelerating structure determination [12,13,15,58]. As a result, for ABC transporters, it is now common to deliver multiple structures per publication, typically, with different substrates and inhibitors [7,23,25,26,30,47,54], or in different conformations [8,25,51,53,55] (Fig. 1). In essence, cryo-EM has dramatically increased the chances of obtaining an ABC-transporter structure and shrunk the obstacles to decipher multiple conformations or nucleotide states, providing a broader view and deeper mechanistic insights, which raises the impact of the research.

Many conformations within one sample

In cryo-EM single-particle analysis [59], multimodel classifications [60] allow to decipher conformational and compositional heterogeneity within the sample [13,61,62]. Therefore, the particles do not have to be trapped in a single conformation, but instead can also be imaged under active turnover conditions. This allows assessing their conformational spectrum and, likewise, to follow their reaction to various compounds. In the past, before the resolution revolution [1], such studies were limited by the signal and accuracy of the electron detection system and the amount of particle images that could be obtained. Accordingly, the analyses were restricted to negative-stain EM, where the obtained signal-to-noise ratio is significantly better; however, at the cost of imaging the imprint of a dried sample, using a heavy-metal solution. If done right, negative-stain EM is a robust, quick, and simple method; however, the precise assignment of the conformations in an ABC-transporter sample is not unambiguous by this technique, mostly due to the possible misinterpretation of conformation and orientation of individual particles. Therefore, more elaborated workflows, involving random-conical tilt [63], are necessary. Such a workflow allowed to describe conformational spectrum of several ABC transporters [64], albeit only at domain resolution, prohibiting interpretation at atomistic levels. In modern cryo-EM, it is now possible to perform such experiments in solution and at resolutions equivalent to those of X-ray crystallography, as shown for the heterodimeric ABC-transporter TmrAB [8]. In this study, TmrAB was imaged under active turnover conditions, which, combined with trapping of transient intermediate states, allowed to decipher the full conformational cycle of the transporter. Fundamental to this study was the ability to acquire large datasets involving millions of particles, as, despite the high fidelity of electron detectors, sufficient number of particles need to be obtained to get enough signal for each conformation. For the statistical analysis, the
Fig. 1. Overview of ABC-transporter cryo-EM structures. Only cryo-EM structures with a nominal resolution of better than 6 Å and deposited models are included. Resolution, origin, nucleotide state, ligand binding, hydrophobic environment, and EMDB identification number are reported next to each of the 68 maps depicted herein.
relative occurrence of each observed conformation can be directly related to its approximate lifetime. The states, that are not detected in the dataset, either do not exist under the given conditions, or their lifetime is too short and the accumulated particle number is insufficient to obtain an interpretable 3-D reconstruction. The latter assumption holds the promise that, by increasing the size of the dataset, more states can eventually be identified. In essence, this would mean that a well-setup cryo-EM experiment under turnover conditions could deliver not only multiple high-resolution structures, but the statistical analysis of their relative appearance would further provide insights into the dynamics, dwell times, and energy landscapes. Through the analysis of bound nucleotides and substrates, the various conformations could be sorted in accordance with their nucleotide hydrolysis and substrate transport state—a new way of performing qualitative ATPase activity and substrate transport assays on the grid.

### Challenges in statistical analysis

However, in cryo-EM, it is not always simple to obtain robust statistics, as the entire particle population has to be accurately represented within the

| Table 1. Advantages and limitations of cryo-EM for analysis of difficult targets and conformational dynamics together with possible solutions. |
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| **Advantages** | **Limitations** | **Possible solutions** |
| Difficult targets (multisubunit, fragile, and native complexes) | No requirement for crystals | Low signal-to-noise ratio | Hardware improvement: higher sensitivity cameras, energy filters, and laser phase plates [103] |
| Imaging in vitrified suspension | Preferred orientation of the particles | Dissociation of complexes and particle destruction | Software improvement: motion correction, micrograph denoising [58,104] |
| Imaging under turnover conditions—many conformations in one sample | Complicated statistical analysis | Missing spatio-temporal information | Sample modifications: antibody technology to increase sample size [105,106] |
| Applicability to heterogeneous and flexible samples | Low sample consumption | High concentration | New sample preparation methods [67–72, 109] |
| Studies in native membranes | Medium resolution, low signal-to-noise ratio, particle size | Usage of cryoprotectants, like glycerol | Tilted-data collection [107] |
| Compatiblity with various hydrophobic environments | Usage of cryoprotectants, like glycerol | Minimizing additives in the buffer | Different grid treatment and different detergents [74,108] |
| Conformational dynamics | High tolerance to minor sample modifications—trapping in various conformations, ligand binding studies | Low throughput | Graphene grids and other support films [75,84,85] |
| Imaging under turnover conditions—many conformations in one sample | Complicated statistical analysis | Missing spatio-temporal information | Improved sample preparation methods [67–72] |
| Applicability to heterogeneous and flexible samples | Resolution and molecular weight | Software improvement [61,62,110,111] | Improved particle picking and multimodel classification algorithms [58,61,65,66] Integrative approaches |
| Antibody technology [105,106] | | Antibody technology [105,106] | |
dataset. For single-particle analysis, this poses a tremendous obstacle, as particles are iteratively discarded during processing. Most of the times, the rejected particles are broken or simply do not provide enough high-resolution signal, but even good quality particles are at high risk to be discarded and left out of the equation, if they belong to under-represented conformations. How or whether discarded particles have to be integrated into the statistical analysis is currently unclear. Additionally, particle picking algorithms still result in significant numbers of false positives and negatives, which skews particle statistics. Furthermore, individual conformers may exhibit different stability and some may be more vulnerable to the relatively harsh cryo-EM specimen preparation than others. The combined effect of these various problems remains unexplored and poses a major challenge for the statistical analysis. The ongoing optimization of both sample preparation and particle picking algorithms should already lead to more robust statistics. For example, neuronal network-based particle pickers [58,65,66] allow to minimize false positives and, moreover, implement micrograph denoising algorithms to enable detection of low-signal particles. Gentler cryo-EM specimen preparation methods [67–72] are expected to reduce the number of damaged particles. In combination, this should result in a cleaner and healthier pool of particles—whether this is really the case, still needs to be investigated.

Caveats of cryo-EM sample preparation

Another source of errors can be found in the specimen preparation itself (Table 1). To enable imaging of hydrated ABC transporters in high vacuum and also to protect them from radiation damage, they need to be vitrified. This process is complex and gives rise to two major concerns. First of all, cryo-EM imaging requires a thin specimen, which is typically achieved by applying the sample onto a hydrophilic object carrier, blotting it to a thin film and rapidly flash cooling it in liquid ethane for vitrification. This approach is not only wasteful, as the vast majority of the sample is disposed, but it can also disintegrate the proteins. The composition of the filter paper and its contact with the sample [73], as well as the extensive interaction of the proteins at the air–water interface, is most detrimental [74]. Especially, the air–water interface is well documented to cause severe sample damage [75] and, furthermore, leads to preferred particle orientation hindering 3-D reconstruction. The approaches to overcome the caveats in cryo-EM sample preparation are reviewed in [76] and new ways of sample preparation, without the need of conventional blotting, are in development [67–72] to minimize sample damage. Ultimately, such technology will be crucial to allow a more robust analysis of the conformational distribution in the dataset.

Additionally, it is currently unclear to what extent the flash cooling in liquid ethane affects particle dynamics. On the order of macromolecular time scales, the rapid plunge-freezing may be not fast enough to avoid a temperature shock of the protein and could, therefore, affect the observed conformational spectrum, which would skew the interpretation. How vitrification affects ABC transporters is currently unclear and cannot be answered without additional experiments. To address this, it would be necessary to systematically assess the effects of vitrification by an integrative approach, using identical samples. A similar strategy would be necessary to obtain the spatio-temporal information, which is missing in cryo-EM experiments. To compensate for that, methods for time-resolved cryo-EM [72,77–80] are developed; however, integrative approaches, combining cryo-EM with single molecule techniques, seem to be the easiest way to obtain such information for now.

Cryo-EM and low-abundant ABC transporters

Structural analysis of native proteins and multiprotein complexes

Cryo-EM provides a unique opportunity to study low-abundant native samples, purified from endogenous sources. While high-affinity tags are absent in such preparations, utilization of tagged inhibitors and substrates, monoclonal antibodies or nanobodies offer a facile approach to pull endogenous complexes out of the cell. This strategy allows to stay close to native conditions and to overcome the common challenges of overexpression in recombinant systems, which often prevent the analysis of multiprotein assemblies, like it has been the case for the human MHC-I peptide loading complex (PLC) [28]. In this study, the PLC was purified from its native environment, using a tagged viral inhibitor as bait, for structural characterization. Using multimodel analysis, several assembly states of the complex were detected in the sample, providing valuable insights into the biological function of the PLC.

While multimodel classifications allow to compensate for compositional heterogeneity, multiprotein complexes are often fragile and tend to dissociate,
which limits the applicability of this approach. In such cases, where sample quality is the limiting factor, simply expanding the dataset may not be a solution. To improve stability of the complex, it can be treated by cross-linking [81] or other biochemical methods, as it was done in the case of the MacAB-TolC ABC-type tripartite multidrug efflux pump [27], which spans the entire Escherichia coli cell envelope and is embedded into two membranes.

The yields of endogenous or multisubunit complexes are typically low, but cryo-EM sample preparation is compatible with comparably small volumes. Recent developments in cryo-EM specimen preparation [67–72], involving picoliter dispensing, reduce the required sample amounts even further and alleviate the burden of large-scale protein production. In the near future, it is very likely that picoliter dispensing is going to become routine, which will open entirely new perspectives on sample preparation and the ability to study low-abundant targets and rare complexes.

**Challenges in obtaining sufficient particle concentration**

While picoliter dispensing significantly reduces sample volumes, concentration requirements are increased when compared to the traditional blotting-based approach. The reasons for this are not entirely clear, but are likely connected to reduced interactions of the protein at the air–water interface and the absence of blotting-related concentration effects [82]. Therefore, to obtain sufficient number of particles on the grid, the purified protein typically needs to be concentrated, which is not optimal for fragile membrane protein complexes that often do not survive this process. Cross-linking renders the sample sturdy enough to withstand the concentration procedure [28]; however, at the same time, it inactivates the protein complex, which may not be desirable. Since standard cross-linking in solution may lead to sample aggregation and other artifacts, gentler cross-linking procedures, like gradient fixation (GraFix) [81] or agarose fixation (AgarFix) [83], have been developed specifically for cryo-EM sample preparation.

To increase the concentration and, additionally, delimit particle interactions with the air–water interface, the sample can be suspended on a thin support layer that spreads across the object carrier. As pristine graphene is invisible to the electron beam, it does not raise any additional background and is, therefore, the ideal choice of support layer, especially for small proteins like ABC transporters. The generation of pristine graphene is currently very challenging and limits its broad usage in the field; however, many efforts are put in optimization of graphene grids [84–86] and, therefore, the situation is likely to change soon.

**Cryo-EM structure determination of ABC transporters in lipid environments**

**ABC transporters in native environment**

Obtaining high-resolution structures of proteins and their complexes within their cellular environment has been a long-awaited dream of many scientists. With cryo-electron tomography (cryo-ET) and subtomogram averaging this vision is now becoming true for large or highly symmetric targets. For example, the HIV-1 capsid-SP1 was imaged to 3.9 A resolution [87], and recent software developments allowed to visualize a drug-bound ribosome inside the cell at 3.7 A resolution [88]. For ABC transporters, however, high-resolution in situ studies are hampered by their relatively small size and low abundance in the membrane. Significant technological developments will be necessary to overcome this limitation. Nevertheless, where high resolution is not absolutely necessary, in situ analyses of multisubunit complexes are already feasible as demonstrated for the MacAB-TolC-related RND-type tripartite multidrug efflux pump AcrAB-TolC [89].

Imaging in native lipid environment can also be achieved by usage of fragmented cells, liposomes, or budded extracellular vesicles (ECVs). ECVs from mammalian cell culture provide an especially appealing option for small proteins like ABC transporters. Combined with subtomogram averaging, ECVs were already been shown to be applicable to in situ characterization of proteins below 100 kDa [90]. Altogether, this demonstrates the potential to study ABC-transporter complexes within their native environment, albeit, currently, at resolutions that are insufficient to accurately build models.

**Compromises for in vitro systems**

In an ideal experiment, the supporting hydrophobic shell accurately represents the native lipid environment, resembling not only the specific composition of the various lipids but also additional factors, such as the lateral pressure and membrane curvature. However, for in vitro systems this cannot be fulfilled. For single-particle analysis, the transporter has to be isolated from the cell and stabilized as a single unit, therefore, the simulation of an ideal membrane is close to impossible and compromises have to be made.
Traditionally, the cells are solubilized using a mild detergent that preserves the structural integrity of the complex. For ABC transporters, digitonin seems to be a popular choice [6,19–23,29,32–34,47,48,52,91,92] (Fig. 2). After solubilization, the detergent can also be exchanged into alternatives, such as amphipols or nanodisks. Of note, co-purification of annular lipids is commonly encountered, even when the protein is treated with detergent. However, it is desirable to keep the environment as close to native as possible and avoid the usage of detergent. In this regard, styrene/maleic acid (SMA) [41] and diisobutylene/maleic acid (DIBMA) [93] copolymers provide an especially beneficial alternative to membrane protein solubilization. While SMA lipid particles (SMALPs) have been shown to be suitable for structural studies in general, their application for ABC-transporter studies is rather limited due to incompatibility with divalent ions, like Mg^{2+}. SMA derivatives, like styrene maleimide—quaternary ammonium (SMA-QA) polymers [94], which display higher stability in the presence of divalent metal ions and large pH range, is, therefore, a better alternative. Another good substitute in this regard is DIBMA lipid particles, but the applicability of these two systems for high-resolution structural studies still needs to be proven. While the full repertoire of detergents, nanodisks and various polymers can be used to stabilize the transmembrane domains of ABC transporters [35,37,39,40,95–97] for cryo-EM, the choice of the hydrophobic environment remains sample-specific and various systems should be screened to best preserve structural integrity and activity, and yield high-resolution structures.

Environment-dependent bias

ABC transporters seem to be very sensitive to the composition of their hydrophobic environment. As such, their ATPase activity is typically significantly reduced in detergent, when compared to liposomes or
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Cryo-EM in ABC-transporter drug discovery

Substrate and inhibitor binding studies

ABC transporters are of high clinical relevance as they are responsible for multidrug resistance in bacteria and cancer cells and are also associated with various diseases, including cystic fibrosis, hypercholesterolemia, Stargardt disease, and other retinal dystrophies [99]. Extensive efforts are, therefore, directed toward development of specific ABC-transporter inhibitors and modulators. Typically, such drug-discovery studies are aided by X-ray crystallography; however, human ABC transporters are challenging targets to crystallize, raising high interest in cryo-EM for substrate/inhibitor binding studies. By now, cryo-EM can contribute comparative studies of the same transporter under different conditions, it is insufficient to explain the molecular triggers that cause conformational bias on its own. Integrative approaches are, therefore, necessary to evaluate the interdependence of activity, conformational spectrum, and the hydrophobic environment. To fully address this phenomenon, in situ analyses are required.

High resolution of small particles

For a long time, the potential of cryo-EM in structure-based drug design (SBDD) has been considered to be low due to the limitations in resolution, molecule size, and low throughput; however, the situation is rapidly changing and expectations from the pharmaceutical industry are increasing. SBDD, typically, requires resolutions in the range of 2.5 Å or better [100]. Given the current resolution record of 1.2 Å for an ideal test specimen [16,17], it is now demonstrated that cryo-EM has the potential to achieve sufficiently high resolutions for SBDD. However, the sample remains the limiting factor in terms of high resolution. Compared to test samples, the additional complexity of ABC transporters, comprising a more demanding sample preparation, the requirement for an ideal hydrophobic environment, their large flexibility, and relatively small molecular weight, is currently limiting similar results. The latter two points pose the biggest challenges in cryo-EM, where the signal that can be deducted from the micrographs is one of the main factors limiting the achievable resolution. To alleviate size-related challenges or to stabilize a transporter in a particular conformation, antibodies can be used. Such technology has been shown to enable high-resolution characterization of as small membrane proteins as, for example, 50-kDa malaria parasite transporter [18] and has also been successfully applied to several ABC transporters [8,24,54]. On the other hand, resolutions in the 3.1–3.5 Å range for ABC transporters are now becoming common even without such fiducials [19,22,23,26,42,44–47,49,51,56,91,92]. Therefore, it is preferable to avoid antibodies whenever possible to minimize the risk of locking the transporter in a non-physiological conformation.

Recently, cryo-EM structures below 3 Å have been reported [8], indicating that structure-based drug design is within reach for these flexible proteins. Given the rapid developments in the field, higher resolutions are to be expected in the near future. However, care must be taken when interpreting cryo-EM maps, as the computed resolution as a sole readout is
insufficient to judge the quality of the reconstruction. This is a well-known problem in the cryo-EM field, causing numerous discussions.

**Low throughput**

For antibody epitope mapping projects, cryo-EM has been shown to be especially useful [101]. However, in SBDD, where many drug–protein complexes need to be characterized in a short period of time, the throughput is still insufficient. Despite the increasing speed of direct electron detectors, improvements in microscope hardware and the application of beam-image shift for data collection [15], which significantly reduced data acquisition time, cryo-EM still cannot nearly compete with X-ray crystallography in terms of throughput. Furthermore, the limited access to high-end microscopes prevents the broad application of cryo-EM in SBDD. Nevertheless, the foundation has been laid and once many synchrotron-like cryo-EM facilities are established the situation is likely to change. Altogether, this yields high expectations for cryo-EM application in SBDD in the near future, especially, for ABC transporters that are not approachable by X-ray crystallography.

**Conclusions and perspectives**

The staggering amount of high-resolution structures obtained by cryo-EM (Fig. 1) over the past few years indicates that cryo-EM has become the method of choice for most ABC transporters, even those that have been successfully crystallized. Indeed, cryo-EM provides not only high-resolution structures but also enables multiple new approaches to analyze these highly dynamic machines. Further advances in data processing will permit to outline the conformational spectra with even higher fidelity, and to understand the inner workings of ABC transporters in unprecedented detail. Given the increasing importance of cryo-EM for such studies, it is now imperative to evaluate the impact of vitrification and to provide the dimension of time. To address these issues, integrative approaches will be necessary.

New sample preparation strategies will push cryo-EM to the next level. For instance, miniaturization [102] will enable direct analysis of endogenous samples and of native multicomponent complexes, especially those resisting recombinant expression. Cryo-ET and subtomogram averaging are gaining attention from both users and developers, which raises expectations for *in situ* studies of ABC transporters. High-resolution data of membrane protein complexes within native membranes, therefore, represent the major goal for future experimental design and research.

Equally important is to increase the throughput of cryo-EM to facilitate streamlined SBDD and to accelerate screening and sample optimization. Likewise, more cryo-EM facilities have to be established to provide access to high-end electron microscopes without the burden of the significant financial investments.

Despite its tremendous success and wide applicability to many research topics, cryo-EM alone cannot and will not provide an answer to everything, but it supplies researchers with a versatile arsenal of tools and empowers them to look at ABC transporters from a new angle.

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