Current outcomes when optimizing ‘standard’ sample preparation for single-particle cryo-EM

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Summary

Although high-resolution single-particle cryo-electron microscopy (cryo-EM) is now producing a rapid stream of breakthroughs in structural biology, it nevertheless remains the case that the preparation of suitable frozen-hydrated samples on electron microscopy grids is often quite challenging. Purified samples that are intact and structurally homogeneous – while still in the test tube – may not necessarily survive the standard methods of making extremely thin, aqueous films on grids. As a result, it is often necessary to try a variety of experimental conditions before finally finding an approach that is optimal for the specimen at hand. Here, we summarize some of our collective experiences to date in optimizing sample preparation, in the hope that doing so will be useful to others, especially those new to the field. We also hope that an open discussion of these common challenges will encourage the development of more generally applicable methodology. Our collective experiences span a diverse range of biochemical samples and most of the commonly used variations in how grids are currently prepared. Unfortunately, none of the currently used optimization methods can be said, in advance, to be the one that ultimately will work when a project first begins. Nevertheless, there are some preferred first steps to explore when facing specific problems that can be more generally recommended, based on our experience and that of many others in the cryo-EM field.

Introduction

When prepared as well-dispersed particles for high-resolution cryo-electron microscopy (cryo-EM), biological macromolecules are ideally embedded in a film of vitrified buffer that is not much thicker than the particle itself. The standard method for preparing such specimens is to apply excess sample to a holey-carbon support film, blot away most of the excess sample and vitrify the remaining thin film by plunging it into liquid ethane. As diagrammed in Figure 1, this idealized picture implies that the condition of particles within the thin layer of vitrified buffer is almost identical to what it was within the test tube. This assumption, in turn, implies that the preparation of electron microscope grids of frozen-hydrated specimens (cryo-EM grids) should be successful every time, for every specimen, at least in those areas of the EM grid where the vitrified ice is thin enough, but not too thin.

The reality is that preparation of samples for cryo-EM can fail in at least four generic ways (Drulyte & Johnson, 2018), even when the condition of macromolecules within the test tube is excellent. The problems encountered in practice include: (1) preferential orientation of particles may occur within thin films (Tan et al., 2017; Noble et al., 2018; D’Imprima et al., 2019), (2) unexpectedly low numbers of particles may be found within holes, i.e. many fewer than is expected from their concentration in bulk solution (Meyerson et al., 2014; Snijder et al., 2017), (3) particles may disintegrate within thin aqueous films and (4) unexplained aggregation of sample material may be observed. It is evident that the idealized cartoon of what happens when cryogrids are made, as shown in Figure 1, is not the complete story.
Interaction of particles with the air–water interface is the most likely cause of problems that emerge when making extremely thin films of sample on grids, but not otherwise, i.e. not in bulk solution (Glaeser & Han, 2017). The denaturation of proteins at gas–liquid interfaces was observed many decades ago (Donaldson et al., 1980), and it is common advice that air bubbles should be avoided even when handling proteins in bulk solution. These denaturing effects must also be present when making thin films of sample on EM grids. Cautions about interactions with the air–water interface were already mentioned in the earliest papers describing how to vitrify thin films (Dubochet et al., 1988). The issue was raised again in the context of estimating that diffusion will result in approximately 1000 or more collisions per second with the air–water interface when the sample thickness is 100 nm thick or less (Taylor & Glaeser, 2008). Nevertheless, it is only recently that cryo-electron tomography has been used to show that adsorption to the air–water interface often leads to preferential orientation of particles (Noble et al., 2018; Chen et al., 2019), and that partial (D’Imprima et al., 2019) or full disruption of particles can occur.

As has recently been reviewed (Glaeser, 2018), denaturation of proteins at the air–water interface has long been studied in other fields, and thus, it is not surprising that it also causes problems when making cryo-EM samples. Indeed, what is perhaps surprising, is the fact that many proteins seem to survive when they are adsorbed to the air–water interface, even when they are preferentially oriented (Noble et al., 2018), although that is not always the case (D’Imprima et al., 2019). In general, large, symmetric structures such as virus particles and filamentous assemblies, often appear to be more resilient, and bacterial proteins and complexes are also generally more robust when prepared as cryo-EM specimens than are their eukaryotic counterparts.

Several ideas and approaches have already been developed to optimize the outcome when the preparation of cryo-EM grids proves to be difficult (Drulyte & Johnson, 2018). But while any given method, such as adding detergent to the sample, or chemically crosslinking the particles, may work for some ‘difficult’ macromolecular complexes, the same method may not work for others. As a result, the current situation in the field is that one must empirically try a number of such methods, one after the other, without knowing in advance which, if any, will succeed.

We here present a set of examples that reflects our experience with preparing cryo-EM grids, which covers work spanning a diverse range of biological macromolecules. Our goals in presenting these are (1) to share our collective estimates regarding how frequently the preparation of cryo-EM grids actually proves to be quite difficult and (2) to gather, in one place, a number of examples in which a given optimization method worked well for one type of specimen but not for another. In addition, we recommend that the previous critique of outcomes published by Drulyte & Johnson (2018) be read together with what we add here.

Not surprisingly, efforts are currently under way to develop better solutions for preparing yo-EM grids, some of which are being pursued in our own respective laboratories (Han et al., 2016; Razinkov & Dandey, 2016; Dandey et al., 2018; Liu et al., 2019). In the interim, although these efforts are under way, this compendium may make it easier for others to get a broader view about the all-too-frequent number of cases where preparing yo-EM grids proves to be difficult.

Our experiences are presented in three sections. The first section consists of a narrative synthesis of the responses of the participating authors to a survey questionnaire. This questionnaire covered issues such as the frequency with which preparing grids did or did not require optimization; the nature of the challenges that had to be overcome for samples that proved to be difficult; and examples in which a given optimization approach did – or did not – succeed. The second section presents representative images that show examples of unsatisfactory results obtained when preparing grids for cryo-EM, and the third section presents examples in which a given optimization approach finally did produce the desired improvement.

### Synthesis of survey results

A multiple-choice questionnaire, covering five topics and three classes of specimen, was first circulated to participating authors. They were asked to individually identify, for each question, the one response that most closely matched their own experiences, rather than what they had heard colleagues say. The full questionnaire, and the tabulated results, are included as part of Supporting Information.

Although responses to this questionnaire are necessarily based on imprecise estimates, and to some extent they may be
considered to be anecdotal, the premise behind this methodology is that the combination of many such estimates is more likely to describe what can be expected to happen, than does an estimate made by any single individual – see, for example, https://en.wikipedia.org/wiki/The_Wisdom_of_Crowds.

In brief, there is overwhelming consensus that (1) optimization is required, much more often than not, for the way in which each kind of sample is prepared for cryo-EM, and (2) none of the currently used methods can be identified in advance as being one that will work. Although we expect that few will disagree with this consensus, we nevertheless believe that it is valuable to further elaborate on these two points.

Success on the first few attempts is rare; optimization is needed more often than not

It was generally felt that successful preparation of cryo-EM grids, during the first few attempts, happens less often than 25% of the time. Three authors actually felt that the success rate may be less than 10% of the time for soluble macromolecules, whereas one author felt that the success rate for icosahedral particles and helices might be as high as 50%. The consensus opinion is that the chance of success (without extensive optimization) is perhaps somewhat better for solubilized membrane proteins than it is for soluble macromolecules.

All participants responded that all four types of unwanted behaviour enumerated in the Introduction can be expected both for soluble macromolecular complexes and for detergent-solubilized membrane proteins. The four problems of preferential orientation, too few particles, disintegration of particles and aggregation of particles were mentioned less often for icosahedral particles and helices, however.

The optimization methods that are currently used within the cryo-EM community include varying the buffer composition (pH, ionic strength, etc.) (Chari et al., 2015); adding small-molecule ligands, substrate molecules or inhibitors; adding macromolecular binding partners or antibodies; creating intramolecular crosslinks with glutaraldehyde (Kastner et al., 2008, Mei et al., 2018) or BS3 (Anand et al., 2018; Kasinath et al., 2018); adding detergents or other surfactants; and adsorption to a support film such as graphene oxide (Pantelic et al., 2010) or even evaporated carbon. Less commonly used optimization methods include applying the sample to holey grids two or more times (with washes in between separate applications) (Snijder et al., 2017); exposing grids to a glow discharge in vapour of amylamine (Fonseca et al., 2015; Nguyen et al., 2018); eliminating the step of treating grids with a glow discharge (Shen et al., 2015); optimizing the blotting conditions such as the time, blotting force, pause between blotting and plunging, ambient temperature and relative humidity; and use of manual blotting (Herzik et al., 2017, 2019) rather than an automated machine.

When optimization is required, the consensus opinion is that approximately 10 different methods, or combinations of methods, have to be tried for soluble macromolecular complexes before one is found that works well. Responses on this point were more varied for detergent-solubilized membrane proteins and for icosahedra and helices, however.

None of the existing optimization methods works consistently well for different kinds of specimens

The addition of detergent or, in the case of membrane proteins, another surfactant such as nanodiscs (Gao et al., 2016) or amphipol (Liao et al., 2013) was the most frequently mentioned method that resulted in successful preparation of cryogrids. However, because trying different surfactants is a common strategy, it was also the most frequently mentioned method that did not produce a successful result.

Although optimizing the buffer conditions was reported to be successful for some particles, it was mentioned even more frequently as being something that did not help. Adding a substrate, an inhibitor or another ligand was mentioned as being successful about as often as it was said to not result in success. Crosslinking with glutaraldehyde or with BS3 was mentioned more frequently as being successful than otherwise. Including an additional macromolecular binding partner was also mentioned more frequently as a method leading to success than it was said to have led to no improvement. Although applying sample two or more times, the use of evaporated carbon as a support film and the use of graphene oxide as a support film are all methods that did work well for some specimens, these were the most frequent ones to be mentioned as not giving a successful result. Similarly, not exposing the grids to a glow discharge was mentioned quite frequently as not resulting in improvement.

The participating authors were next asked to identify up to five cases in which some of the above methods either succeeded or failed. A subset of examples, both successes and failures, was selected from the many responses. The number selected for publication was limited to 2 for each lab, so as to not put too heavy a burden on the preparation of figures for publication by the students or postdocs involved in the original work, who are acknowledged in the figure legends.

Examples of cases in which a particular optimization method was not effective

Figure 2 shows six examples of what various problematic samples look like when the results after the first few trials to prepare yo-EM grids failed, and it was recognized that further optimization needed. Problems encountered in the initial screens included: extensive particle aggregation when thin films were prepared on EM grids (Fig. 2A), preferential orientation of nicely dispersed particles that had otherwise looked very promising (Figs. 2B and 2D), clumping of filamentous particles (Fig. 2C) and disintegration of particles into small pieces (Figs. 2E and 2F). Results of initial optimization attempts
included binding immunoglobulin G (IgG) antibodies or IgG-derived Fab fragments (Fig. 2A), having detergent present in the sample buffer (Figs. 2B and 2C), and chemical crosslinking with either glutaraldehyde or BS3 (Figs. 2E and 2F).

Turning to the complete survey results reported in Table S1 of the Supporting Information, five responses cited cases in which either using graphene oxide support films or adding detergent or another surfactant did not improve the situation; four reported cases in which optimizing the buffer, optimizing the type and concentration of small-molecule ligand, performing chemical crosslinking, applying sample two or more times or using continuous carbon as the support film, each did not improve the situation; and two cited cases in which adding a macromolecular binding partner did not solve the problem. Although each of these methods failed for some of the samples, they nevertheless were effective for others, as will be discussed in the following section.
Fig. 3. Six examples of what various samples look like after nontrivial optimization has led to improvement. (A) HIV-1 envelope trimmers in complex with monoclonal Fab fragments at two separate antigenic sites (Chuang et al., 2019); scale bar indicates 100 nm. This is an example in which the use of additional macromolecular binding partners not only overcame the problem of aggregation that occurred on the grid, but, in addition, this made it possible to obtain a 3D reconstruction at sub-nm resolution. Figure provided by Dr. Priyamvada Acharya. (B) The calcium-activated chloride channel, TMEM16A, again purified in LMNG, but now Fab fragments have been bound at two independent sites; scale bar indicates 20 nm. The addition of antibodies improved the distribution of Euler angles, but the average resolution of the 3D map obtained with these particles was worse than without the antibodies. Nevertheless, a better, more interpretable map resulted when data were combined for particles with and without bound antibodies, Figure provided by Dr. Shangyu Dang. (C) Filaments of a complex formed between Dynamin-Related Protein 1 (DRP1) and Mitochondrial Dynamics Protein 49 (MDP49) (Kalia et al., 2018); scale bar indicates 50 nm. The addition of 0.2% octyl glucoside detergent substantially relieved the clumping seen in Figure 2(C). Figure provided by Dr. Raghav Kalia. (D) The Csy complex shown in Figure 2(D) was subsequently bound to a double-stranded DNA oligomer target and vitrified in a buffer containing 0.05% LMNG; scale bar indicates 100 nm. The addition of a macromolecular cofactor, DNA in this case, overcame the problem of preferential orientation. Figure provided by Dr. Saikat Chowdhury (Rollins et al., 2019). (E) Chemically crosslinked Polycomb Repressive Complex 2 (PRC2) on continuous carbon film, with 0.01% NP40 detergent added (Kasinath et al., 2018). Although these particles now look good, the sample still suffers from preferential orientation. Figure provided by Dr. Vignesh Kasinath. (F) Exocyst complex (Mei et al., 2018); scale bar indicates 100 nm. Less aggressive crosslinking than that used for Figure 2(F), in this case using 0.0025% glutaraldehyde, was effective in preserving particles when on the grid. Figure provided by Yan Li.

Examples of cases in which optimization methods led to significant improvement

Figure 3 shows six examples of what some samples looked like after nontrivial optimization that was at least partially successful. Methods include binding of Fab fragments (Figs. 3A and 3B); addition of detergent (Fig. 3C); addition of a macromolecular binding partner (Fig. 3D); a combination of binding to continuous carbon film, chemical crosslinking and addition of detergent (Fig. 3E); and using less aggressive chemical crosslinking (Fig. 3F).

Turning again to the complete survey results reported in Table S1, five responses cited success in optimizing grid preparation by adding some type of detergent or other surfactant and five improved the grids by adding a small-molecule inhibitor, substrate, or other ligand; four improved their results by using some form of chemical crosslinking; three
had success after adding a macromolecular binding partner and three had success after applying sample to the grid two or more times; whereas only two found that further optimization of the pH, ionic strength, etc., was effective. Although each of these methods were effective for some proteins, they had nevertheless failed for some other proteins, as was described in the previous section.

Based on our combined experience at this point, we suggest a few common-sense actions as the ones to consider taking first, should optimization of grid preparation be required. The suggestions given below assume that the particles appear to be homogeneous in size and shape when in negative stain, to a resolution of perhaps 15 Å or 20 Å. If that is not the case, one may have to reconsider whether the particles are, in fact, well behaved in the test tube, i.e. before the step of making cryo-EM grids.

**Preferential orientation**

Try any, or even a combination of, the following: (1) adding detergent, (2) adding Fab fragments or IgG antibodies or (3) adding an additional macromolecular binding partner. It is also worthwhile to try binding the particle to a very thin, continuous support film such as glow-discharge treated, evaporated carbon films or perhaps graphene oxide. Finally, it is worthwhile to try using holey-gold on gold grids (Russo & Passmore, 2014), rather than holey carbon on copper grids. The use of holey-gold support films makes it practical to record images at moderate tilt angles, thereby increasing the angular distribution of particles with respect to the incident beam (Tan et al., 2017).

**Particles do not go into holes**

One recommended action is to again try binding the particles to very thin, glow-discharge treated evaporated carbon films or perhaps graphene oxide. Because adsorption to a continuous support film such as glow-discharge treated evaporated carbon films is likely to result in concentrating the particles on the grid, such measures may be necessary if only very small amounts of protein are available. When the amount of sample is not a limitation, however, one can try to apply sample two or more times (Snijder et al., 2017).

**Particles are broken or disintegrated**

Crosslinking with glutaraldehyde or BS3 is the best option to try in this case.

**Particles are badly aggregated**

Try adding detergent or another surfactant. This is especially recommended if aggregation is already seen in negatively stained samples.

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**Summary and conclusions**

It is not uncommon to get poor results when preparing grids for single-particle cryo-EM; only rarely does grid preparation succeed during the first few tries. This commonly occurring issue need not be because the biochemistry has not yet been optimized, or because the investigator did not know how to make grids (although either could contribute to the problem). As a result, extensive optimization is often required, even by those who have had considerable previous success with other samples.

Several orthogonal optimization methods, enumerated here, have each proved to be effective for at least some particles. Any one method nevertheless does not work equally well for all macromolecular particles. As a result, optimization of the method used to prepare cryogrids requires the empirical testing of many different ideas, without knowing in advance which, if any, is likely to succeed.

This compendium presents examples of both successes and failures for some of the more commonly used optimization methods. The goal is that our experiences, as well as those of other work cited here, may serve as a starting point for others, should the preparation of cryogrids prove to be difficult for a new particle of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supporting Information