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Technical Note

Know your detergents: A case study on detergent background in negative stain electron microscopy

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ABSTRACT

Electron cryo-microscopy (cryo-EM) of purified macromolecular complexes is now providing 3D-structures at near-atomic resolution (Kühlbrandt, 2014). Cryo-EM can tolerate heterogeneous specimens, however, high-resolution efforts demand highly optimized samples. Therefore, significant pre-screening and evaluation is essential before a final dataset can be obtained.

While cryo-EM is comparably slow and requires access to expensive high-end electron microscopes, room temperature negative stain EM is fast, inexpensive and provides immediate feedback. This has made it a popular approach for sample quality control in the early phases of a project. Optimization in negative stain can be critical not only for cryo-EM, but also for X-ray crystallography, as highlighted for example by studies on GPCR complexes (Kang et al., 2015; Rasmussen et al., 2012). However, when not done carefully and interpreted correctly, negative stain can be prone to artifacts. A typical problem, which is often overlooked in the interpretation of EM data of small membrane proteins, is the background, caused by empty detergent micelles, as it can be easily confused with detergent embedded protein samples.

To counteract this ubiquitous problem, we present a case study on commonly used detergents. We show that most detergents produce significant background in negative stain EM, even below nominal critical micelle concentration (CMC). Unawareness of such artefacts can lead to misinterpretation of sample quality and homogeneity. We hope that this study can serve as a template to evaluate images in the early phases of a project.

1. Optimization of membrane protein preparations in negative stain EM

In cryo-EM purified samples are imaged at liquid nitrogen temperature in vitrified suspension. This allows the usage of a broad spectrum of hydrophobic environments, including various detergents, amphipols, beta peptides, saposin nanoparticles or lipid nanodiscs, to stabilize membrane proteins and their complexes (Frauenfeld et al., 2016; Nath et al., 2007; Popot, 2010; Tao et al., 2013). This versatility, in combination with recent technological advances (Kühlbrandt, 2014), has led to an increase of high-resolution structures of membrane proteins, including TRP channels, ABC transporters or G-protein complexes, which were previously extremely challenging targets for structural studies (Liao et al., 2013; Oldham et al., 2016; Shukla et al., 2014; Zhang et al., 2017).

High-resolution cryo-EM and single particle analysis, especially, of difficult targets, requires samples of best quality. For membrane proteins, it is important to realize that different buffer components may affect the obtained contrast, and some detergents can be directly visible in cryo-EM (Hauer et al., 2015; Vinothkumar and Henderson, 2016). In many cases, negative stain EM has shown to be an important asset towards ideal sample quality. Here, sample preparation is comparably easy and only very little amounts and concentrations are needed. At intermediate magnification a large field of view is achieved, which enables robust statistical assessment of the sample from a small number of micrographs. Therefore, negative stain EM can be used as a high-throughput approach, especially, when combined with automated data acquisition routines (Lyumkis et al., 2010). High-resolution details are not resolved by negative stain EM, primarily due to the grain size of the staining agent and structural information is limited to the surface of the protein. Additionally, artifacts may arise from sample drying, flattening or the low pH of the stain solution. However, these circumstances only need to be taken into account for high-resolution approaches, as for sample optimization, the focus is on general information about sample quality. Negative stain EM is a priceless method for analyzing sample homogeneity, aggregation, complex formation or the effects of different buffer compositions, especially, in the case of membrane proteins, where sample amounts are often limited and cryo-EM is complicated

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due to the small size (Blees et al., 2017; Kang et al., 2015).

2. Detergent background in negative stain EM

While it is widely acknowledged that sample preparation for cryo-EM requires experience and extensive training, negative stain EM is often considered to be simple and easy. However, if not done carefully, it can be prone to incorrect interpretation. A typical problem that arises from negative staining is a considerable amount of background when membrane proteins are being imaged. In some cases, this effect is so severe that alternative methods have to be devised to mute it (Hauer et al., 2015). If possible, empty micelles should be generally avoided, however, this is typically not trivial and the workload to do this in the early stage of a project for every individual sample may rapidly overcome the benefits of high-throughput screening. A membrane protein with sufficient extra-membrane mass that resides on the support film in an orientation, which allows for an unambiguous assignment, can be easily spotted in negative stain despite a significant background. In practice, if the structure is unknown or if insufficient features are present, it is often not so trivial and empty micelles can be easily confused with protein, which can lead to severe misinterpretation of the data. Therefore, it is essential to be aware of the background, caused by detergents, especially during optimization of small membrane protein samples. Here, we present a case study on 14 commonly used detergents (Lyons et al., 2016) and show the imprints of the corresponding micelles at different detergent concentrations, which should serve as an atlas to quickly asses what kind of background can be expected from a given detergent.

3. Negative stain imprints of the detergent micelles

For our experiments, detergents were stained with uranyl formate, following well established protocols (Tao et al., 2013). In all cases, 3 µl of detergent sample were applied onto freshly glow-discharged carbon-coated G400-C3 grids (Gilder Grids), blotted from the side and stained by applying three times 3 µl of 2% uranyl formate and blotted from the same side after each application. The data was recorded on a Tecnai™ Spirit 12G TEM (ThermoFisher – former FEI), operating at 120 kV and equipped with a GATAN 4096 × 4096 CCD detector by automated data collection with the Leginon software package (Suloway et al., 2005). Images were collected at a nominal magnification of 42,000, corresponding to a pixel size of 2.68 Å (nominal defocus – 1.50 µm). For imaging, areas with pristine staining were chosen as normally done for protein samples (Januliene et al., 2017; Moeller et al., 2015; Yang et al., 2015).

Fig. 1 illustrates a typical problem in negative staining of membrane proteins: the comparison between the fully membrane embedded, ~100 kDa, dimeric citrate symporter CitS (pdbid: 5A1S (Wöhlert et al., 2015)) in DDM/CHS (Fig. 1A) and the DDM/CHS buffer alone (Fig. 1B) immediately visualizes how easy it can be to misinterpret empty micelles with the protein. In both cases, the micelles are similar in diameter, shape and contrast and even the computation of 2D class averages (Scheres and Chen, 2012) cannot resolve protein from the empty micelles. While negative stain analysis of CitS in DDM/CHS is impossible, a detergent exchange to DM (Fig. 1C), with its minimal background (Fig. 1D), resolves this issue. Here, the symporter can be clearly visualized and comparison between 2D class averages and simulated versions, obtained from the crystal structure, are in appropriate agreement (Fig. 1E).

As demonstrated in Fig. 1, some detergents are more suitable for structural studies than others, based on the produced background. Therefore, to help to assess various backgrounds, we imaged 14 different detergents, that are frequently used in structural studies to stabilize membrane protein complexes (Lyons et al., 2016). To standardize the experiments the same low salt buffer was used for all detergents (20 mM HEPES pH 7.0, 100 mM NaCl) at three different concentrations (the detergent concentrations were calculated based on the theoretical CMC values in water, as provided by the vendor (Table S1)), in the absence of protein. In Fig. 2 we show the imprints of these detergents at 5xCMC, 1.1xCMC and 0.7xCMC. Above CMC most of them produce significant background, displaying many micelles of different size and shape. The number of micelles, their size and heterogeneity vary in a concentration dependent manner. Interestingly, the structural appearance of the detergents can be fundamentally different and not all micelles are spherical. For example, LMNG appears as worm-like filaments, which could be easily confused with filamentous protein structures. Some detergents cover the entire carbon support film at high concentration (e.g. NG and DPC), which render them almost unusable for negative stain EM of small proteins. It is commonly known that the addition of CHS enlarges the micelle size (Thompson et al., 2011) and this can also be directly seen in our data. Both DDM and C12E8 exhibit a much stronger background and also larger micelles when mixed with CHS, which renders it impossible to detect an embedded small membrane protein. Some of the better examples are DM and digitonin, where the number and diameter of the micelles is in principle acceptable for structural studies. Surprisingly, while reduced, the background is still obvious below the theoretical CMC in most of the samples.

From our experience not only detergents will add to the background but also other frequently used buffer components. Therefore, to complete the study we further provide the background of amphipols (A8-35), empty nanodiscs, as well as the imprint of glycerol.

4. Final considerations

Availability and imaging time on high-end electron microscopes is limited and should not be wasted. Negative stain EM provides an efficient method to rapidly pre-screen samples on lower end machines, which are generally more accessible. Of note, such sample screening can be utilized not only for cryo-EM studies, but it is also beneficial for crystallization trials (Kang et al., 2015; Rasmussen et al., 2012).

Nevertheless, the user must be aware of certain important pitfalls that are connected to this approach. Beyond the typical effects of staining on carbon support films (flattening and preferred orientation) different buffer components can significantly add to the background. This is extremely obvious when detergents are used, as presented in this study.

Small membrane proteins with little extra-membranous domains can be easily confused with empty micelles. Also, a high background might disguise larger membrane proteins at an early stage, when particle concentration and homogeneity has not yet been optimized. To counteract such problems, we suggest the following strategies:

1) To visualize the background, the buffer should be imaged in the absence of a protein.
2) Additionally, the sample should be always imaged at least at two different concentrations to judge whether features originate from protein or buffer components.
3) If single particle analysis is hindered by a particular detergent, exchange to another detergent with lower background is recommended.

Our data suggest that most detergents will be visible in negative stain EM already below their nominal CMC. Therefore, it can be generally stated that detergents produce significant background under typical experimental conditions. Unawareness of this will lead to misinterpretation of sample quality and homogeneity. We hope that this study will serve as a basis to compare different membrane protein preparations and that it will support the use of high-throughput negative stain.