Higher resolution in cryo-EM by the combination of macromolecular prior knowledge and image-processing tools

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Single-particle cryo-electron microscopy has become a powerful technique for the 3D structure determination of biological molecules. The last decade has seen an astonishing development of both hardware and software, and an exponential growth of new structures obtained at medium-high resolution. However, the knowledge accumulated in this field over the years has hardly been utilized as feedback in the reconstruction of new structures. In this context, this article explores the use of the deep-learning approach deepEMhancer as a regularizer in the RELION refinement process. deepEMhancer introduces prior information derived from macromolecular structures, and contributes to noise reduction and signal enhancement, as well as a higher degree of isotropy. These features have a direct effect on image alignment and reduction of overfitting during iterative refinement. The advantages of this combination are demonstrated for several membrane proteins, for which it is especially useful because of their high disorder and flexibility.

1. Introduction

Single-particle analysis of macromolecular structures by electron cryo-microscopy (cryo-EM) has been established as a key technique in structural biology, with the power to reach near-atomic resolutions and explore conformational flexibility. Thousands or even millions of projections of the macromolecule of interest in different orientations (2D images obtained in the microscope) are used to determine its 3D structure. Different algorithms have been developed for the 3D reconstruction process from the images (Grant et al., 2018; Grigorieff, 2007; Ludtke et al., 1999; Punjani et al., 2017; Scheres, 2012a; Sorzano et al., 2018), in which the orientation and position of the particles in each image are inferred by comparing them with in silico projections of a reference map. However, the images acquired at the microscope are extremely noisy, which make the search for the correct angular assignment of the particles a challenge.

One of the most established reconstruction approaches in the field is based on iterative refinement formulated as maximum a posteriori (MAP) optimization (Punjani et al., 2017; Scheres, 2012a, 2012b), although the practical use of rich priors in these methods is very limited, which we will comment on later. At each iteration, the angular assignment of each particle is updated, while the new maps obtained are modified (by regularization) to suppress noise and thus reduce overfitting. The most commonly used strategy to avoid overfitting is a regularizer in the form of a space-invariant filter, which is
applied equally throughout the space and is determined by the Fourier shell correlation (FSC) (Chen et al., 2013; Harauz & Heel, 1986; Rosenthal & Henderson, 2003; Scheres, 2012a; Scheres & Chen, 2012). However, these filters do not consider the spatial heterogeneity of the maps (i.e., different regions may have different resolutions), although approaches have been developed to use local filtering after refinement (Cardone et al., 2013; Kucukelbir et al., 2014; Vilas et al., 2018), achieving more reliable results.

Other solutions have been presented with the purpose of mitigating local overfitting. In cryoSPARC, the non-uniform refinement algorithm (Punjani et al., 2020) introduces an adaptive cross-validation regularization which is applied at each iteration of the refinement. In the case of RELION (Scheres, 2012a), the new external reconstruction functionality (Kimanius et al., 2021) allows investigation of ways to include the introduction of priors through a script that runs external software to modify the intermediate maps without regularization. This functionality is used by SIDESPLITTER (Ramlaul et al., 2020), which relies on a modified adaptation of the LAFTER algorithm (Ramlaul et al., 2019) to denoise intermediate maps during refinement.

Currently, deep-learning-based methods have a high impact on cryo-EM and are being used in different stages of processing such as denoising (Bepler et al., 2020), particle picking (Wagner et al., 2019; Wang et al., 2016), map reconstruction (Gupta et al., 2021; Zhong et al., 2020) or local resolution estimation (Ramírez-Aportela et al., 2019). Indeed, the idea of using tools based on deep learning to denoise the intermediate maps within the iterative process of density-map refinement has also been raised (Kimanius et al., 2021), which would allow us to introduce previously acquired knowledge about biological macromolecules to the refinement process. However, this approach was only tested on simulated datasets and has not been applied to experimental data.

In this paper we present the first development in which rich, protein-specific, prior information derived from experimental information deposited in public databases is applied to experimental data for alignment. We incorporate this prior information through the use of our recently introduced deep-learning approach deepEMhancer (Sanchez-Garcia et al., 2021), which is applied iteratively within RELION. deepEMhancer performs a non-linear transformation of the volume that produces a new density map which incorporates EMDB-related prior information resulting in masking/denoising, sharpening effects as well as a higher degree of isotropy. Naturally, this additional information is expected to be especially useful in difficult cases in which parts of the map are affected differently by flexibility/local disorder or local noise, as is typically the case for membrane proteins embedded in lipid bilayers. We would also expect that increased map isotropy would help in extracting the most from samples presenting preferred orientations, rather than a possible exacerbation of this problem during refinement. Our test clearly shows substantial enhancement of the results when compared with standard RELION and SIDESPLITTER.

2. Methods

2.1. deepEMhancer in relion_refine

In order to facilitate better treatment of the signal during the reconstruction of the maps and avoid overfitting, deepEMhancer was integrated into the iterative process of relion_refine. It was previously shown that deepEMhancer boosts the signal of the map (sharpening effect) while at the same time producing a noise reduction effect. Also, deepEMhancer incorporates prior information from macromolecules acquired during its deep-learning process. These benefits were further demonstrated in membrane proteins: suppressing most of the signal coming from the lipid layer and enhancing the signal belonging to the protein (Sanchez-Garcia et al., 2021).

Since version 3.1, an external reconstruction functionality was enabled in the RELION refinement program (Kimanius et al., 2021). When the --external_reconstruct argument is used, relion_refine waits while an external program modifies the unregularized half-maps at each iteration. In this work, this new functionality is used by deepEMhancer to enhance the signal and remove noise from the intermediate reconstructions. deepEMhancer is applied independently to each of the generated half-maps. However, it is only executed in the last iterations, when the process enters the local angular search. For this purpose, the variable rhnHealpixOrder in the relion_iter_sampling.star file is monitored at each iteration.

2.2. Reconstruction using simulated datasets

Initially, the proposed refinement protocol was studied using simulated data. The first case was based on the structure of β-galactosidase (PDB entry 3j7h; Bartesaghi et al., 2014). A density map with a sampling rate of 0.637 Å and a box size of 338 × 338 × 338 was derived from the structural model, calling the function xmipp_volume_from_pdb (Sorzano et al., 2015) from the Xmipp package (de la Rosa-Trevín et al., 2013; Strelak et al., 2021). Using this map, projections were generated in all directions with an angular sampling of 1.5°, for a total of 18,309 projections. Gaussian noise with zero mean and a standard deviation of 150 was added to the set of projections (as shown in Fig. S1 of the supporting information, where images with noise of different standard deviations are presented). The projections were then used for the unmasked ‘3D auto-refinement’ in RELION while the initial map was taken as a reference.

The second case tested was based on the structure of the 20S proteasome (PDB entry 6bdf; Campbell et al., 2015). A similar protocol to the previous one was followed. However, in this case the map was created with a box size of 256 × 256 × 256, a sampling rate of 1.0 Å and the set of projections generated was anisotropic, increasing projections in the cone formed between tilt angles of 0 and 40°. A total of 24,359 projections were generated and processed for refinement. In both cases RELION (version 3.1) was used and no solvent-mask was provided.

In a third case, attempting to get closer to a real experiment, 18,309 projections generated from the reconstructed map
using the EMPIAR-10391 dataset were added to ‘pure noise’ particles picked over the deposited micrographs. Micrograph CTF estimation was performed using GCTF (Zhang, 2016). JANNI (Wagner & Raunser, 2020) was used to denoise and facilitate picking in areas where there was no particulate matter. For noise particle selection we use the Xmipp particle-picking algorithm (Abrishami et al., 2013). The extracted particles were subjected to two rounds of 2D classification using cryoSPARC (Punjani et al., 2017) to discard particles with macromolecular signal. Finally, 18,309 ‘pure noise’ particles were selected. To study the effect of noise level, the intensity of the selected particles was increased by 5, 10 and 15×, respectively.

Table 1
Validation metrics for different reconstructions of β-galactosidase.

| Resolution (Å) | RELION | SIDESPLITTER | deepEMhancer |
|----------------|--------|--------------|---------------|
| 3.12           | 3.03   | 2.87         |
| Q scores       | 0.772  | 0.780        | 0.789         |
| FSC-Q (Å)      | 0.37   | 0.37         | 0.33          |

2.3. Experimental datasets

Three datasets were obtained from EMPIAR to test the applicability of deepEMhancer within the RELION refinement: EMPIAR-10391 (Tan et al., 2020b), EMPIAR-10254 (Dang et al., 2019) and EMPIAR-10420 (Tan et al., 2020a). All refinements were computed from particle image stacks, with refinements were carried out through RELION. The resolutions achieved by each method are shown in Table 1. The best results were obtained using deepEMhancer. In addition, Fig. S2 shows the map obtained using deepEMhancer superimposed on the starting atomic model, where the high level of agreement can be appreciated. Note these results indicate that the reconstructed map which best represents the ground truth model is the one obtained using deepEMhancer, indicating our proposed method has not introduced systematic artifacts in the maps.

Further analysis on the control of the appearance of artifacts was conducted by filtering the particles at different resolutions and varying the noise levels before refinement. First the particles were low-pass filtered at frequencies of 3, 5 and 8 Å with a raised cosine of 0.0064 (in discrete frequency normalized to 0.5), while in all cases Gaussian noise was added with zero mean and 150 standard deviation (SD). This implies that the data generated do not present frequencies above approximately 2.92, 4.76 and 7.40 Å. Fig. S3 shows the behavior of the FSC curve for the different reconstructions using standard RELION or in combination with deepEMhancer. The resolution achieved using deepEMhancer was slightly higher in all cases than using standard RELION, but in no case did the resolution exceed the cutoff frequency.

The dependency on the level of noise was tested in a second experiment in which different levels of Gaussian noise were added (with 50, 200, 400 and 1000 SD; Fig. S1) to the set of particles filtered to 5 Å. Fig. S4 shows the FSC curves for the reconstructed maps. In the case of a very high noise level (corresponding to the case of noise with 1000 SD), the HEALPIX variable does not exceed 2, so in this case deepEMhancer is not applied in any iteration and the resolution achieved is the one of standard RELION (17 Å; data not shown in the supporting figures). In Fig. S4 we observe that, with increasing noise, the resolutions achieved using standard RELION decrease, whereas this effect is less accentuated in the case of introducing deepEMhancer.

The second case corresponds to the known structure of the 20S proteasome (PDB entry 6bdf). In this test, the set of projections generated was not homogeneous, simulating the occurrence of preferred directions. Some previous work has shown an ‘attraction’ problem in RELION when the data have an over-abundance of projections in certain directions (Sorzano et al., 2021, 2010). This test was designed to allow us to study the effect of deepEMhancer when there were important differences in the number of images along the different projection directions. Using these data, the resolu-
Table 2
Validation metrics for different reconstructions of 20S proteasome.

| Resolution (Å) | RELION | SIDESPLITTER | deepEMhancer |
|---------------|--------|--------------|--------------|
| Q scores      | 0.284  | 0.410        | 0.481        |
| FSC-Q (Å)     | 4.06   | 2.01         | 0.75         |

Refinement of synthetic data generated from the 20S proteasome structure (PDB entry 6bdf). Three different refinements were made using standard RELION, using SIDESPLITTER or incorporating deepEMhancer.

3.2. Results on experimental datasets

Having verified the advantages of using deepEMhancer in reconstructions with simulated data, we tested its applicability with experimental data. Three datasets were obtained from EMPIAR: EMPIAR-10391, EMPIAR-10254 and EMPIAR-10420. The cases studied correspond to membrane proteins, because this class of structure is the most likely to benefit from regularizers that consider the spatial non-uniformity of the reconstructions.

3.2.1. Structure of arabinofuranosyltransferase

In a first study using experimental data, the dataset of arabinofuranosyltransferase (AftD) (EMPIAR-10391) was used. AftD contains a membrane-embedded portion with 16 transmembrane helices and a soluble periplasmic portion (Hoffmann et al., 2008). A total of 37,814 particle images of AftD in lipid nanodiscs were used for 3D reconstruction in RELION. For comparison, three reconstructions were performed using standard relion_refine, SIDESPLITTER and deepEMhancer. Figs. 2 and S9 show the density maps obtained by each method. Though the map obtained with SIDESPLITTER is higher quality than that obtained with standard RELION, the best quality map was obtained using deepEMhancer. Better definition is observed in both the transmembrane helices and the soluble portion. This map achieves a resolution of 2.83 Å by the gold-standard FSC of 0.143 Å, whereas 4.24 Å was obtained with standard RELION and 3.99 Å with SIDESPLITTER [Fig. S7(a)]. The improvement is also reflected in the local resolution histograms determined by deepRes (Ramírez-Aportela et al., 2019) [Fig. S8(a)]. The median local resolution was 4.12 Å using deepEMhancer, 4.96 Å with...
SIDESPLITTER and 5.96 Å with standard RELION. We note that there is no additional sharpening operation involved in any of the three results.

An interesting practical question might be posed at this stage about whether the differences presented in these analyses might indeed appear should postprocessing of all the different maps by deepEMhancer be done consistently at the end of the reconstruction process instead of inside the iteration loop. The answer to this question is given in Figs. S11 and S12. Indeed, the differences are less pronounced than with the protocol previously presented, however, we still find areas of lower resolution where applying deepEMhancer within RELION offers significant advantages over using it for postprocessing. Fig. S11(a) shows a fragment of the reconstruction using deepEMhancer within RELION, while panels (b), (c) and (d) show the results after applying deepEMhancer as postprocessing on the maps obtained using standard RELION, or in combination with SIDESPLITTER or deepEMhancer, respectively. In this figure we can see different areas (indicated in red) where better performance is obtained by combining RELION with deepEMhancer. Additionally, and using the multimethod integrative capabilities of Scipion, we can explore this question further and consider the actual angular differences introduced to the refinement process when deepEMhancer is used as an integral part of the iterative process. Fig. S12 compares the differences in spatial shifts (Å) and angular alignment (°) when using three different workflows that are presented throughout this work (the differences between RELION and SIDESPLITTER are shown in dark blue, and the differences between RELION and deepEMhancer are shown in cyan). The differences are to be understood in the context that we are in the same local minima of the optimization process (i.e. they cannot be very large), but they clearly and systematically occur, indicating that, indeed, the iterative use of deepEMhancer has impacted the very essence of the refinement process, as it is the finding of the projection geometry.

3.2.2. Structure of TRPV5. The second case corresponds to the structure of full-length TRPV5 in lipid nanodiscs (EMPIAR-10254). In this case, 87 603 previously deposited particles were used for the reconstructions using C4 symmetry. The three workflows previously described using relion_refine algorithms were run and their corresponding 3D reconstructions are depicted in Figs. 3 and S10. As can be seen, the map obtained with deepEMhancer appears to be better quality, with a clearer definition of the helical pitch in both the region immersed inside the nanodisc and the intracellular domains. deepEMhancer reduces the influence of noise on alignment and produces an improvement in resolution of the protein. Using the gold standard FSC, resolutions of 3.39, 3.35 and 2.15 Å were achieved for RELION, 3.66 Å for SIDESPLITTER and 2.69 Å (deepEMhancer) [Fig. S7(c)]. However, the reconstructed maps present elongations, which are typical of particles with preferred orientations/directions. As discussed previously in the proteasome test, when

![Figure 3](image)

**Figure 3**
Refinements of 87 603 particle images of TRPV5 in lipid nanodiscs (EMPIAR-10254). Comparison of refinement results using standard RELION, SIDESPLITTER and deepEMhancer. No local filtering or sharpening operations were used and the threshold was set to keep the enclosed volume constant. (a) Side view and (b) top view of the reconstructions.

![Figure 4](image)

**Figure 4**
Refinements of 57 970 particle images of EmbB in lipid nanodiscs (EMPIAR-10420). Comparison of refinement results using standard RELION, SIDESPLITTER and deepEMhancer. No local filtering or sharpening operations were used and the threshold was set to keep the enclosed volume constant. (a) Side view and (b) alternative side view of the reconstructions.
performing 3D angular assignment using RELION, some directions might attract particles from other directions, most likely nearby directions. This problem is accentuated in the RELION-only map, although it is also very evident with SIDESPLITTER. Using deepEMhancer we can see that, although the problem persists, the map obtained shows a remarkable correction of this effect. deepEMhancer allows better handling of anisotropic SNR.

4. Discussion

Extreme noise in the images acquired from the microscope may hinder the correct angular assignment of particles during 3D reconstruction in cryo-EM. This issue is even more complex for integral membrane proteins, which are embedded in detergent micelles or lipid nanodiscs and exhibit greater disorder and flexibility. These characteristics increase the spatial variability in the reconstructions, which is reflected in greater SNR heterogeneity. However, traditional reconstruction algorithms in cryo-EM assume spatial homogeneity and the regularizers used to avoid overfitting have also routinely been spatially invariant. Nonetheless, applying a shift-invariant filter may cause noise accumulation in some areas, while in others the signal may be degraded.

Recently, adaptive regularizers have been introduced in reconstruction methods (Punjani et al., 2020; Ramlaul et al., 2020). These regularizers consider the differences in spatial SNR and help to mitigate overfitting. The new RELION functionality (Kimanius et al., 2021) that allows the use of external proposals in the refinement (previously used by SIDESPLITTER) is a very important tool for the development of new hybrid reconstruction methods. This article shows the benefits of integrating deepEMhancer within relion_refine, helping to mitigate overfitting and obtain better quality 3D reconstructions. deepEMhancer introduces information from the macromolecules already learned during the previous training and has a double effect on the intermediate reconstructions; on the one hand, it has a masking/denoising effect, while at the same time enhancing the signal. Furthermore, deepEMhancer takes into account the anisotropic SNRs and improves angular assignment in cases characterized by preferred directions.

Note that deepEMhancer is only applied during local angular assignment in the last iterations of the refinement. This allows the possibility of applying other regularizers during global assignment, such as SIDESPLITTER. However, we found no apparent benefit with the combination of SIDESPLITTER and deepEMhancer in the cases tested. The combination of different regularizers can be evaluated in more detail in future work.

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