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# Focused classification and refinement in high-resolution cryo-EM structural analysis of ribosome complexes

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Cryo electron microscopy (cryo-EM) historically has had a strong impact on the structural and mechanistic analysis of protein synthesis by the prokaryotic and eukaryotic ribosomes. Vice versa, studying ribosomes has helped moving forwards many methodological aspects in single particle cryo-EM, at the level of automated data collection and image processing including advanced techniques for particle sorting to address structural and compositional heterogeneity. Here we review some of the latest ribosome structures, where cryo-EM allowed gaining unprecedented insights based on 3D structure sorting with focused classification and refinement methods helping to reach local resolution levels better than 3 Å. Such high-resolution features now enable the analysis of drug interactions with RNA and protein side-chains including even the visualization of chemical modifications of the ribosomal RNA. These advances represent a major breakthrough in structural biology and show the strong potential of cryo-EM beyond the ribosome field including for structure-based drug design.

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## Introduction

Ribosomes catalyse protein biosynthesis by converting the nucleotide sequence of the messenger RNA (mRNA) into an amino acid sequence. Multiple aiding factors such as tRNAs, initiation, elongation, termination and recycling factors are binding to the ribosome to regulate this process [1–3]. A complication to determine the structure of ribosomal complexes is their flexibility and heterogeneity, due to the intrinsic flexibility of the small and large ribosomal subunits, which show relative and internal conformational changes or may dissociate. Cryo electron microscopy (cryo-EM), in combination with advanced image processing and structure sorting is able to address these issues [4–6]. Therefore, cryo-EM plays an important role in structural and functional studies of ribosomal complexes when studied at high resolution, but it also plays an integrative role at the interface with X-ray crystallography and studies performed in the cellular context such as cryo electron tomography (cryo-ET), focused ion beam and scanning electron microscopy (FIB-SEM) and fluorescence microscopy including super-resolution single-molecule localization microscopy (reviewed in [7]). Recent technological progress in the development of new-generation detectors and improvement of image processing algorithms have greatly enhanced the achievable resolution of structures obtained using cryo-EM [7–9]. Here, we review how these advances in hardware and image processing have helped structural studies of the ribosome as illustrated by several recent examples. Finally, we discuss where we see the next future challenges to be addressed.

## Cryo-EM of ribosomes: direct detectors, advanced image processing and structure sorting to obtain high-resolution features

Direct electron detectors which are based on complementary metal oxide semiconductor (CMOS) devices, such as the Falcon (from Thermo Fisher Scientific/FEI), DE (from Direct Electron) and K2 Summit (from Gatan) cameras, strongly improve the image contrast and the overall signal-to-noise ratio even at high frequencies with enhanced detective quantum efficiency (DQE) values, as compared to charge-coupled device (CCD) cameras or film [10–12]. Thanks to their high sensitivity

even at low electron doses and the fast readout, direct electron cameras allow the acquisition of movies on a given area of the sample instead of a single image; these can be used to correct for local and global movements in the images and also weight the electron dose for optimal transfer of the high-resolution information while limiting the sample irradiation by the electron beam [13–18]; see also review by [7]. The other important advance enabling high-resolution cryo-EM work has been developments in image processing to address sample heterogeneity. These can be either different conformations or distinct compositions (e.g. protein subunits falling off from a larger macromolecular complex), but either of these will limit interpretability and the final resolution of a structure that can be achieved.

Heterogeneous datasets represent a challenge for 3D structure determination because the original concept of 3D reconstruction from 2D projections assumes identical objects seen under different Euler angles. However, the ribosome is a large flexible macromolecular complex, which often leads to the presence of more than one structural state. Thus, advanced classification techniques are required to separate the different conformations in the sample into structurally homogeneous subsets that are amenable to single-particle reconstruction. One can distinguish three different methods (reviewed in [4,6]): first, reference-based techniques like 'supervised classification' (using several related, known structures as starting templates; e.g. [19,20]), second, methods based on statistical analysis [4,21–23] called 'unsupervised classification' that can provide 3D variance maps and can be applied together with random resampling and bootstrapping methods [4,24–28], and third, methods based on maximum likelihood (ML) [29–32] during which random subsets are optimized and a low-resolution average structure is used as reference, that is resampling is used in combination with likelihood optimization.

When classifications are performed in 2D, a basic problem is to distinguish between orientational and conformational classifications [4–6,23]; thus, it is easier to perform structure sorting through 3D classifications [26] and determine at once multiple structures that are in equilibrium with each other. Originally, a first approach to address this consisted in 2D multivariate statistical analysis (MSA) of selected, variable sub-regions of the 2D images (referred to as 'local MSA' [21] or as 'focused classification' [24]) using a re-projected mask for MSA [21] or for cross-validation [24]. This concept of local 2D analysis was then extended to the third dimension, for example 3D resampling and classification (3D-SC) and 3D bootstrapping methods, where a 3D mask can be used for 3D classification and refinement [4,26] (Figure 1a). The concept of focused refinement, that is refining a subregion of a large complex while restraining the movement of the whole complex, was also introduced in ML-based refinements [33] and is now commonly used in the cryo-EM field (e.g. [34–37,38••,39–42]).

Thus, a technological synergy has occurred over the last few years between high-sensitivity cameras and the way cryo-EM images are processed including through particles sorting (Figure 1b). Now, in many instances structures can be determined at a resolution, where atomic details of the structure can be resolved to provide essential insights into molecular interactions of the ribosome with regulatory translation factors and substrates [33–37,38••,39–42,43•,44••,45,46•,47–49,50••,51–54]. For example, the positions and conformations of nucleotide bases and amino acid side-chains of the ribosomal RNAs (rRNA) and proteins can be identified (Figure 1c), along with deriving an atomic model that can be refined against the experimental cryo-EM map [55,56]. In some cases, it becomes possible to visualize fine details like $Mg^{2+}$ and $Zn^{2+}$ ions (Figure 2) and post-transcriptional and post-translational modifications in ribosomal RNA and proteins (Figure 3) [33,34,46•,47,48]. A recent study on a ribosome-bound internal ribosomal entry site (IRES) mRNA from the Taura syndrome virus (TSV) illustrates the strength of particle sorting in cryo-EM [38••]. By separating the different states through image processing the authors managed to resolve the individual snapshots of the IRES translocation through the aminoacyl (A), peptidyl (P) and exit (E) sites of the ribosome, along with the conformational changes of this large mRNA structure containing several folded domains (Figure 1d).

## Inhibitor binding in ribosomes seen by cryo-EM

While the ribosomal core that catalyses peptide bond formation (on the large ribosomal subunit) and mRNA decoding (on the small ribosomal subunit) is universally conserved, the parts of the ribosome involved in translation regulation are less conserved. In fact, 32 of the eukaryotic ribosomal proteins do not exist in bacteria; moreover, archaeal and eukaryotic rRNAs can have more or less pronounced rRNA expansions [44••,57]. Targeting structural and chemical variances between eukaryotic and prokaryotic ribosomes with small ligands is therefore a common approach for drug design against bacterial and fungal infections, where the aim is reaching high specificity to limit side effects. A recent revival is the interest in inherent mechanisms related with cancer for which the human ribosome appears to be a promising target [34,58].

Many ligand complexes of the ribosome have been studied by X-ray crystallography (reviewed by [49]). While it is more convenient to screen ligand complexes by X-ray crystallography because series of ligands can be added through crystal soaking, this limits the choice of the species that can be studied to a handful of microorganisms (e.g. extremophile bacteria, *Escherichia coli*, *Staphylococcus aureus* (reviewed by [49,59,60]) and *Saccharomyces*