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#Protein Lights Up in Cryo-EM

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The molecular weight (MW) threshold of "small" protein molecules approachable by single-particle cryo-EM structural analysis has been constantly revised with the rapid advances in this imaging technique. Before the advent of direct electron detectors, 200 kDa was regarded as the applicable MW limits. Then, with the introduction of phase plates, the 64 kDa hemoglobin was imaged and reconstructed to better than 3.5 Å resolution [1]. Recently, the Lander lab has demonstrated the capability of cryo-EM in structure determination of sub-100 kDa complexes on a 200 keV electron microscope [2], and the Jensen lab has proposed a platform strategy using DARPin for small protein structure determination [3]. Benchmarked on protein molecules with known structure, these achievements also suggest that the potential of single-particle cryo-EM is yet to be fully realized. Utilizing heave elements that provide enhanced amplitude contrast in cryo-EM imaging, we show here that the structure of a 50 kDa copper-storage protein can be visualized at sub-nanometer resolution without resorting to phase-plate imaging. The technical and methodology development will have broader application in EM structural biology.

Methanotrophs require a large amount of copper for particulate methane monooxygenase, for which the supply is facilitated by copper storage proteins (CSPs). In contrast to unstructured apo-metallothioneins that fold around metal clusters, CSPs have the unique capacity for metal ion storage within an established protein-folding motif. The first structure of the CSP protein family, CSP1 from the methanotroph *Methylosinus trichosporium* OB3b, was determined by X-ray crystallography in 2015 [4]. In its native tetrameric assembly, each monomer stores 13 Cu^{+} ions nested within the core of a four-helix bundle. To demonstrate the feasibility of cryo-EM structure visualization on such a small protein, recombinantly expressed and purified Cu^{+}-bound CSP molecules were imaged on a Titan Krios 300 keV electron microscope equipped with energy filter and Gatan K3 camera, but without using phase plates in the data collection. As shown in Figure-1A, the high amplitude contrast of Cu^{+} ions has significantly enhanced the signal in cryo-EM images. A small dataset containing merely 4,050 particles yields a sub-nanometer 3D reconstruction (inset of Figure-1A). Because of the tetrahedron packing of the assembly, some of its 2D projections resemble a "#" sign, therefore the nickname in the title of this article.

Heavy-atom Z-contrast enhancement in cryo-EM imaging offers multiple desirable properties including more reliable particle annotation by computational algorithms for large datasets, better movie-frame registration and particle alignment, and more precise CTF determination as well. In broader structural applications, CSPs can be engineered to serve as a scaffold onto which other proteins bind, thus improving the contrast of the full complex assembly for the structure determination of unknown components. As a proof-of-concept, we utilize the CSP-scaffolding to study the structure of Rho1D4 Fab that has a molecular weight of 44 kDa. The C-terminal 1D4 linear peptide (9 a.a.: TETSQVAPA) is a versatile, multipurpose epitope tag. Together with the Rho1D4 monocolonal antibody, 1D4 can be used to detect and localize proteins in cells by immunofluorescence and immune-electron microscopic labeling, and purify functionally active proteins including membrane proteins by immuno-affinity chromatography. Intriguingly, the structure of Rho1D4 Fab and its epitope interface is unknown (or not in the public domain yet). We have engineered a chimera that fuses the 1D4 epitope tag to the C-terminal of CSP monomer,

and the CSP tetrameric scaffold provides four potential Rho1D4 Fab binding sites (schematics in Figure-1B). To reduce the complexity of conformational flexibility in structure analysis, protein concentration of CSP and Rho1D4 is tuned to 1:1.1 for cryo-EM specimen preparation and data collection. As shown in Figure-1C, the class average has revealed drastic image contrast difference between the CSP scaffold and the Rho1D4 Fab, although their molecular weight are comparable. This pilot study has confirmed the protein design and the rigidity of the chimera protein complex. Its structure elucidation at higher resolution is currently work in progress.

In summary, Z-contrast enhancement in cryo-EM imaging leads to improved image contrast and signal-to-noise ratio. However, because heavy elements have distinctively different electron scattering profiles than light atoms (namely C, N and O) in protein amino acids, the contrast transfer function in TEM imaging deviates from being constant on protein assemblies containing heavy atoms/ions. When the presence of heavy elements is few and sparse, the effect is not readily observable at lower resolution and may be negligible in structure interpretation. In the case of CSP, however, a large number of Cu^{+} ions generate stronger amplitude contrast throughout the protein volume in cryo-EM imaging. In order to push the 3D reconstruction and structure analysis towards atomic resolution, it is imperative to formulate new methods and computational algorithms for proper Z-contrast correction [5].

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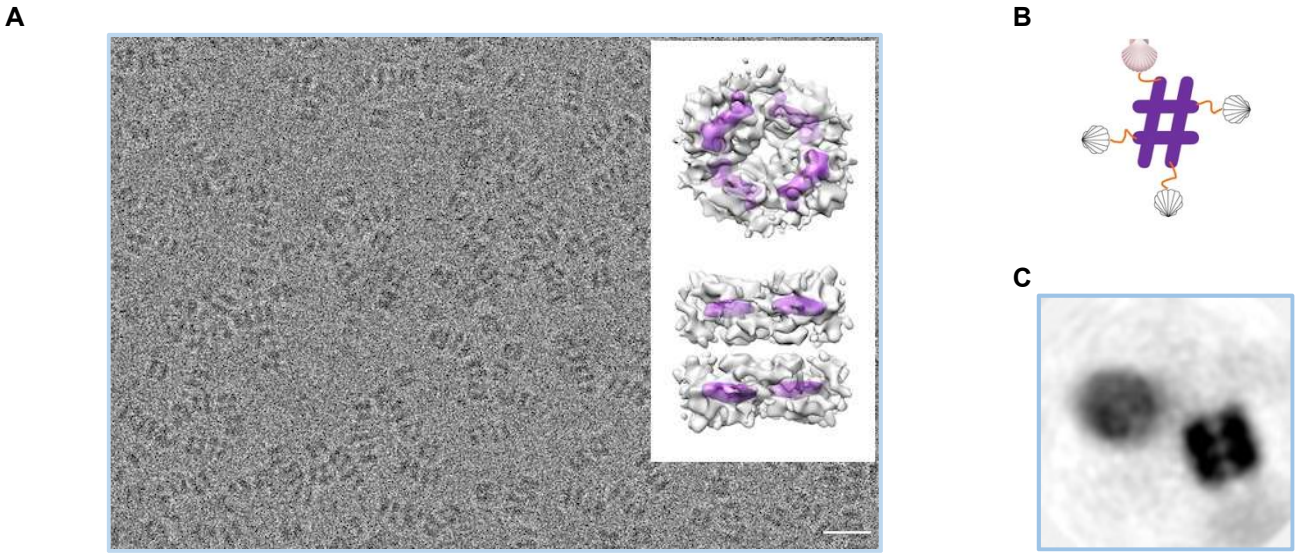

Figure 1. A) Cryo-EM micrograph of CSP particles (*50 kDa, scalebar: 10 nm*) without phase-plate imaging. *Inset:* a preliminary reconstruction, in which the high-density regions associated with Cu^{+}-ions are colored in magenta. B) The concept of CSP ("#") scaffolding for cryo-EM visualization of even smaller protein molecules (short peptide linkers in orange). C) Example: a 2D class-average of the CSP-Rho1D4 chimera complex in cryo-EM, showing significant difference in the image contrast.

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