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Single particle 3D reconstruction for 2D crystal images of membrane proteins

Sebastian Scherer [a], Marcel Arheit [a], Julia Kowal [a], Xiangyan Zeng [b], Henning Stahlberg [a,*]

[a] Center for Cellular Imaging and NanoAnalytics, Biozentrum, University Basel, 4058 Basel, Switzerland
[b] Fort Valley State University, 1005 State University Dr., Fort Valley, GA 31030, USA

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In cases where ultra-flat cryo-preparations of well-ordered two-dimensional (2D) crystals are available, electron crystallography is a powerful method for the determination of the high-resolution structures of membrane and soluble proteins. However, crystal unbending and Fourier-filtering methods in electron crystallography three-dimensional (3D) image processing are generally limited in their performance for 2D crystals that are badly ordered or non-flat. Here we present a single particle image processing approach, which is implemented as an extension of the 2D crystallographic pipeline realized in the *2dx* software package, for the determination of high-resolution 3D structures of membrane proteins. The algorithm presented, addresses the low single-to-noise ratio (SNR) of 2D crystal images by exploiting neighborhood correlation between adjacent proteins in the 2D crystal. Compared with conventional single particle processing for randomly oriented particles, the computational costs are greatly reduced due to the crystal-induced limited search space, which allows a much finer search space compared to classical single particle processing. To reduce the considerable computational costs, our software features a hybrid parallelization scheme for multi-CPU clusters and computer with high-end graphic processing units (GPUs). We successfully apply the new refinement method to the structure of the potassium channel MloK1. The calculated 3D reconstruction shows more structural details and contains less noise than the map obtained by conventional Fourier-filtering based processing of the same 2D crystal images.

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1. Introduction

The low signal-to-noise ratio (SNR) obtained when unstained proteins embedded in a layer of amorphous ice are imaged by cryo-transmission electron microscopy (cryo-EM) hinders their structural analysis. Both the localization and orientation of smaller molecules and complexes are difficult to define precisely, and the use of averaging methods to enhance the signal is correspondingly difficult. The situation can be improved for membrane proteins as they can be incorporated in a lipid bilayer and crystallized in two dimensions (2D). This strongly restricts the orientational freedom of the macromolecule in the crystal lattice. The regular arrangement lends itself to averaging techniques and, of course, to electron crystallography.

Perfect 2D crystals are flat, distortion-free and, unfortunately, almost impossible to obtain. Rather, deviations have to be recognized and corrected for, as do possible imaging effects. A series of image processing and electron crystallography software packages tackle this problem (reviewed in (Arheit et al., 2013b)), the most famous being the MRC programs (Crowther et al., 1996). These have allowed atomic models to be determined for different membrane proteins. The basic procedure used to process crystal images is comprised of six steps: (i) lattice determination, (ii) determination of the tilt geometry, (iii) lattice correction (unbending), (iv) contrast transfer function (CTF) correction, (v) data averaging for each image, and (vi) merging and lattice line fitting of the data from several images in three dimensions (3D). The *2dx* software (Gipson et al., 2007) implements a user-friendly graphical interface to the MRC programs, and adds several additional modules for automation and additional algorithms. *2dx* offers user guidance

Abbreviations: 2D, two dimensions/dimensional; 3D, three dimensions/dimensional; CM, center of mass; CNDBs, cyclic nucleotide binding domains; cryo-EM, cryo-electron microscopy; CTF, contrast transfer function; FSC, Fourier shell correlation; GPU, graphic processing unit; GPGPU, general-purpose graphic processing unit; MPI, message passing interface; SNR, signal to noise ratio; VSDs, voltage sensor domains.

* Corresponding author. Address: Center for Cellular Imaging and NanoAnalytics (C-CINA), Biozentrum, University Basel, WRO-1058, Mattenstrasse 26, CH-4058 Basel, Switzerland.

E-mail address: henning.stahlberg@unibas.ch (H. Stahlberg).

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and facilitates the project management. The conventional crystallographic image processing approach addresses locally disordered crystals by *crystal unbending*, where small image patches (typically 25×25 pixels) are shifted in the image plane. In the present study we show that the mathematical model behind unbending fails if crystals are disordered and tilted, or are non-flat. In order to overcome these limitations, we introduce a new refinement process that resolves local crystallographic disorder in 3D.

Cryo-electron microscopy (cryo-EM) single particle analysis (Frank, 1975) is a technique used to determine the structure of proteins without any crystallization. Randomly oriented single particles are imaged under the electron microscope leading to different views of the protein under investigation. The orientation of the recorded projections is initially unknown. The approximate orientation of each particle is determined by comparing the particles with a set of trial projections obtained from an initial model for instance obtained by *random-conical tilt reconstruction* (Radermacher, 1988). Back-projecting all particles into a 3D volume based on their updated orientations leads to an improved 3D model. This iterative process is repeated until the 3D model no longer changes. A broad variety of single particle reconstruction software packages is available today, for instance: EMAN (Ludtke et al., 1999), BSoft (Heymann, 2001), Xmipp (Sorzano et al., 2004), Sparx (Hohn et al., 2007), Frealign (Grigorieff, 2007) Relion (Scheres, 2012), or Simple (Elmlund and Elmlund, 2012).

Here we present a single particle-based reconstruction method for 2D crystals that is implemented as an optional extension of the *2dx* software. The approach applies the principle of single particle processing to 2D crystals, exploiting neighborhood correlation between adjacent proteins in the crystal in order to overcome the limitations originating from the low SNR. High performance computing hardware is used to handle the computational costs of the new procedure. The major differences of the new method to classical single particle analysis are the new local averaging step, the significantly finer angular search space sampled during the refinement and a new crystal-based particle selection procedure validating the alignment parameters of each particle.

2. Approach

In the MRC programs, the mathematical model behind local 2D correction of crystal disorders by in-plane shifting of small crystalline patches, does not cover all the possible crystal imperfections present in a 2D crystal. 3D rotational disorder is not addressed at all. The simplification of the unbending approach is partially valid for non-tilted specimens but not for tilted specimens (Fig. 1). 3D disorder is parameterized by three Euler angles and two in-plane translational parameters, and must be considered for each protein of a 2D crystal individually to fully exploit the data and achieve reconstructions with the best possible resolution.

Below we introduce a single particle-based reconstruction method for 2D crystals that refines the orientation locally for each particle, while using the correlations of neighboring particles in the crystal to overcome limitations imposed by the low SNR of cryo-EM images. The new processing approach (Fig. 2) is a refinement of the conventional crystallographic image processing pipeline described by (Arheit et al., 2013a,b) (Fig. 2A), and is implemented as an option in the open source *2dx* (version 3.5.0) software. This new procedure can be applied to any previously recorded 3D cryo-electron crystallography dataset.

The initial stages of the classical approach (Fig. 2A) include determination of image defocus and astigmatism with the program *CTFFIND3* (Mindell and Grigorieff, 2003), lattice definition, and correction for translational disorder in the crystal, i.e., image unbending. The latter involves cross-correlation of the raw image with an iteratively improved reference image to define the position of individual unit-cells using the MRC program *Quadserch*, and translational adjustment of small crystal patches using the MRC program *CCUnbend*. Finally, the structural data from all unit cells of one crystal image are combined into one resulting unit cell image at a much higher SNR. The ensemble of data from multiple crystals at different specimen tilts is merged into one 3D reconstruction (MRC programs *Origtilt*, *Latline*, *and others*).

Our new refinement procedure (Fig. 2B and C) uses the results obtained by the classical method as a starting point, i.e., unit cell positions, CTF parameters of each image and the final 3D model,

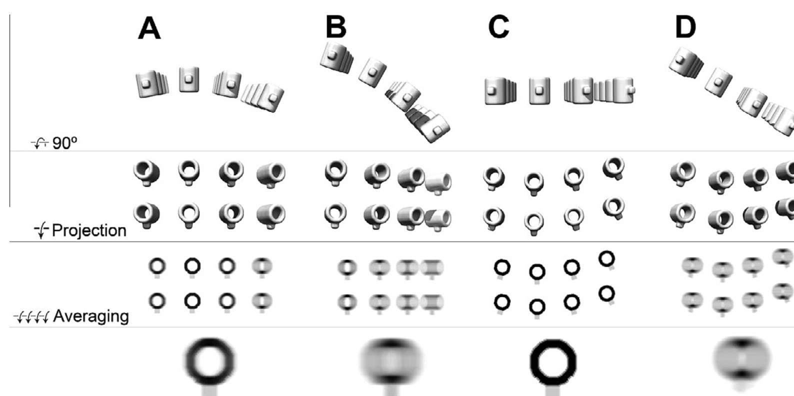

Fig.1. Effect of sample flatness and local disorder on resolution in crystallographic processing. Artificial crystals composed of multiple copies of a tube with a protrusion are considered. From top to bottom: perspective side-view of the crystal; top-view of the crystal (only two protein rows shown for the sake of clarity); the orthographic projection recorded by a transmission electron microscope; the outcome of an optimal crystal unbending procedure as could be achieved by the classical crystallographic processing. (A) Nominally non-tilted, yet locally bent 2D crystal with perfectly in-plane aligned particles. Although the local disorder cannot be retrieved perfectly by unbending the crystal, the merged projection map (bottom row) resembles the true projection of the structure with little resolution loss. (B) The crystal in (A) tilted by 30°. Due to the membrane curvature, the regularity of the crystal is lost in the recorded projection. As a result, high-resolution spots perpendicular to the tilt axis in the Fourier transform of the crystal disappear, which strongly limits the resolution of the projection map (bottom row). Thus the presence of ultra-flat preparations is required when imaging tilted 2D crystals and processing them with the classical lattice unbending approach. (C) Non-tilted perfectly flat crystal with rotational disorder. This is the situation in which the classical unbending procedure performs optimally. Note that even the rotational disorder can be corrected by using an optimal patch size in the unbending step. (D) The perfectly flat crystal in (C) is tilted by 30°. Here, the classical unbending-based processing cannot deal with this situation as the projection image contains multiple different views of the proteins in the crystal. The classical processing fails to align the difference projections from slightly different view points. Taken together, the unbending-based processing performs well on non-tilted crystals, but encounters serious limitations for tilted crystals, even if they are perfectly flat but rotationally disordered.

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