High-resolution cryo-EM using beam-image shift at 200 keV

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ABSTRACT

Recent advances in single-particle cryo-electron microscopy (cryo-EM) data collection utilizes beam-image shift to improve throughput. Despite implementation on well-aligned 300 keV cryo-EM instruments, it remains unknown how well beam-image shift data collection affects data quality on 200 keV instruments and whether any aberrations can be computationally corrected. To test this, we collected and analyzed a cryo-EM dataset of aldolase at 200 keV using beam-image shift. This analysis shows that beam tilt on the instrument initially limited the resolution of aldolase to 5.6Å. After iterative rounds of aberration correction and particle polishing in RELION, we were able to obtain a 2.8Å structure. This analysis indicates that software correction of microscope misalignment can provide a dramatic improvement in resolution.

Keywords: single-particle cryo-EM; beam tilt; RELION; aldolase

INTRODUCTION

In order to increase the throughput from cryo-EM instruments, many laboratories and facilities have begun using beam-image shift for data collection (Cheng et al., 2018). Using this approach, instead of moving the stage to each position on the cryo-EM grid, a process that requires precise movement, the beam is moved in conjunction with image adjustments. Without long waiting times of moving the stage, tilting the beam leads to a dramatic increase in the number of exposures per hour. As such, it is now routine to use beam-tilt to collect 100-300 exposures whereas previously it was only possible to collect 40-50 per hour. This throughput will continue to increase with the advent of direct detectors with faster frame rates, leading to hundreds of exposures per hour.

Even though users can collect 2-3X the amount of data using beam-image shift, they must overcome an additional aberration induced by the beam-image shift: beam tilt (also called ‘coma’) (Glaeser et al., 2011). When using beam-image shift for collecting exposures, the resulting image will have beam tilt, an aberration that will dampen high-resolution (<3Å) information in the micrographs (Glaeser et al., 2011). Due to this, it is a common practice to minimize beam tilt in the cryo-EM instrument through microscope alignments ahead of data collection.

Beam tilt aberrations can be corrected computationally for high-resolution structures. For example, this was implemented by Henderson and coworkers for the atomic-resolution structure of bacteriorhodopsin from 2D crystals (Henderson et al., 1986). Since its use 40 years ago, recent advances in single-particle cryo-EM have led to the incorporation of beam tilt correction into software packages such as RELION (Herzik et al., 2017; Zivanov et al.; Wu et al.). The availability of beam tilt correction has led to its widespread adoption for cryo-EM structure determination.

Typically, users are finding 0.2-0.8 mrad beam tilt on previously aligned 300 keV Titan Krios
where $\theta$ is the value required to return instrument to a coma-free state, $\lambda$ is electron wavelength, $C_s$ is the spherical aberration of the objective lens, $s$ is spatial frequency, $\mathbf{\hat{t}}$ is a unit vector indicating direction of beam tilt, and $\mathbf{\hat{s}}$ is a unit vector indicating the direction of the spatial frequency vector. Equation (1) indicates that changing from 300 keV ($\lambda = 1.96$ pm) to 200 keV ($\lambda = 2.51$ pm) will result in a 1.640X worse phase error from beam tilt. While previous work indicated that short-range beam-image shift can achieve a 3.3Å for the T20S proteasome at 200 keV (Herzik et al., 2017), this same work required using stage position to obtain a resolution better than 3Å. Recently, using these original datasets of aldolase and T20S datasets, RELION-3.1 now allow higher-order aberrations to be corrected computationally (Zivanov et al.; Wu et al.). This allowed the resolution of aldolase to improve from 3.1Å to 2.3Å.

In order to test the limits of computational correction of microscope aberrations at 200 keV, we collected and analyzed a dataset of aldolase using beam-image shift on a Talos-Arctica at 200 keV. In order to mimic a real-life scenario, the microscope was not aligned on the day of data collection but had been aligned within the past week. Using this dataset, we were able to determine a 5.6Å structure of aldolase without beam tilt correction. Following iterative rounds of beam tilt correction and 3D refinement, we were able to achieve a 2.8Å structure of aldolase. This indicates that beam-image shift can be an effective data collection strategy to increase the throughput on 200 keV cryo-EM instruments, where microscope aberrations can be corrected computationally.

**RESULTS**

**Beam-image shift data collection & analysis**

In order to test the impact of beam-image shift on data quality, we set up the automated data collection system to target 5x5 areas with
beam-image shift (Figure 1A). Ahead of data collection, the only microscope alignments performed were beam-tilt pivot points and objective astigmatism. At medium magnification (Figure 1A), we typically focused on the middle hole which was followed by beam-image shift with
distances up to 5 μm away from the beam center. After collecting 2,111 micrographs, we obtained a large range of beam-image shift micrographs that provided a near-continuous distribution across the 10 x 10 μm area (Figure 1B). Interestingly, while many micrographs showed minimal objective astigmatism (Figure 2A, left), a large percentage of the dataset showed exaggerated objective astigmatism (Figure 2A, right) which can be induced by a large amount of beam tilt (Glaeser et al., 2011).

Following data collection, the aldolase beam-image shift data were analyzed using standard single-particle processing (Figure 2). This involved estimating the contrast transfer function (CTF) using CTFIND4 (Rohou & Grigorieff, 2015), which yielded CTF fits to higher than 4Å resolution for the majority of the micrographs (Figure 2B). After picking and extracting particles, 2D classification showed clear secondary structure features (Figure 2C), consistent with previous work on aldolase (Herzik et al., 2017; Kim et al., 2018). After selecting particles from class averages exhibiting high-resolution features, we performed 3D classification in order to obtain a homogenous population of aldolase particles with all four subunits intact (Figure 2D). Using these selected particle coordinates, particles were re-extracted at the full pixel size (0.91 Å/pixel) and subjected to 3D refinement in RELION. The refined structure reached a resolution of only 5.6Å (Figure 2E), which is significantly less than published work of ~3Å (Kim et al., 2018; Herzik et al., 2017). This suggested that the aberrations from beam tilt induced by beam-image shift data collection are likely limiting the resolution of the final structure.

The influence of the beam tilt aberration is also apparent when inspecting the FSC curve for the final reconstruction (Figure 2F). The corrected FSC curve was dramatically attenuated when compared to the uncorrected FSC curve. This is due to the correction applied in RELION, whereby the phase randomized reconstruction is subtracted from the uncorrected FSC curve (Chen et al., 2013). For this reconstruction of aldolase, the phase randomized FSC curve has more signal than the uncorrected FSC curve, leading to dramatic negative values seen in the corrected FSC curve. We believe these differences are caused by the phase differences for the data coming from a wide range of beam tilts.

**Beam tilt correction of aldolase cryo-EM micrographs**

After determining a refined 3D structure of aldolase, we wanted to test whether the beam tilt refinement option in RELION 3.0 is capable of overcoming such a large degree of beam tilt. To use this feature of RELION, the micrographs must be grouped into beam tilt groups. Considering the near-continuously changing beam-image shift data collection for the entire dataset (Figure 1B), beam-image shift values from Leginon were used in order to divide the micrographs into groups (Figure 3A). This involved dividing data into groups of 4 (2 x 2), 25 (5x5), 100 (10x10), and 400 (20x20) based on the amount of beam tilt. For each grouping, the particles underwent beam tilt refinement, 3D refinement, and sharpening in RELION in order to determine the change in final resolution of the structure (Figure 3B). We saw that three distinct result sets: no change (4 micrograph groups), nominal improvement to ~4.5Å (25 and 100 micrograph groups), and maximal improvement 4.2Å (400 groups). This result indicates that the previously determined structure at 5.6Å was limited in resolution due to beam tilt aberrations that could be partially overcome by grouping the data into 400 beam tilt groups in RELION.

For the micrographs divided into 400 groups, the subsequently refined map showed improved density features and had a gold standard FSC value of 4.2Å (Figure 3C & 3D). The FSC curve for the 400 micrograph groups showed an FSC possessing a more typical appearance of FSC curves (Figure 3D), although the shape still
indicated aberrations may be affecting the 3D reconstruction. This indicates that beam tilt refinement improved the resolution of aldolase significantly from 5.6 to 4.2Å in a single step.

Using the calculated beam tilt values from RELION, we then compared how beam tilt changed as a function of microscope beam-image shift (Figure 3E). This comparison reveals a few key features of this dataset. First, without any applied beam-image shift at [0,0], there was a significant amount of beam tilt present: -1.19 mrad (X) and 1.07 mrad (Y). Second, the change in beam tilt based on change in beam-image shift (the slope in Figure 3E) was different for the X

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**Figure 3** - Improved resolution and map quality using beam tilt refinement. (A) Strategy for grouping micrographs. Micrographs were grouped into 4 groups (2x2), 25 groups (5x5), 100 groups (10x10), 400 groups (20x20), or one per micrograph (503 groups). (B) Effect of group size on beam tilt refinement and subsequent resolution estimation for refined 3D structures. (C) Sharpened 3D reconstruction for particles placed into 400 micrograph groups filtered to 4.2Å. (D) FSC curves for 3D reconstruction in (C). (E) Beam tilt measurements for each group displayed with respect to microscope beam-image shift for X (black) and Y coordinates (gray). Dashed lines show least squares fit.
versus Y direction: -2.0e04 μm/mrad vs. 1.23e4 μm/mrad, respectively. Finally, this result also shows that a subset of micrographs have a much larger beam tilt than the majority of micrographs, explaining why some micrographs displayed objective astigmatism due to high beam tilt (Figure 2C).

Given that the RELION beam tilt estimation step is dependent on the resolution of the 3D reconstruction, we performed iterative beam tilt refinements and Bayesian particle polishing in RELION (Figure 4). Starting with the 4.2Å reconstruction (Figure 4B), we used this map to re-calculate beam tilt for micrographs across the dataset. Then, using these new beam tilt values, we performed another round of 3D refinement. This new structure refined to higher resolution at 3.8Å and had a lower B-Factor (-164Å²) (Figure 4C). After these two rounds of beam-tilt refinement, we then utilized Bayesian particle polishing in RELION (Zivanov et al., 2019) to further improve the resolution to 3.3Å (B-Factor -129Å²) (Figure 4D).

Repeated CTF-refinements and particle polishing allowed us to determine a final structure of aldolase at 2.8Å (B-Factor -55Å²) (Figure 4I, Figure 5A, Supplemental Figure 1). This structure shows dramatically improved density features compared to the original 5.6Å structure (Figure 5B). Specifically, the significantly higher resolution provides unambiguous secondary structure tracing whereas the 5.6Å structure contained many more ambiguities (Figure 5B). Comparison of model refinement statistics also highlights the improve map quality for the final 2.8Å reconstruction (Supplemental Table 2). The FSC curve for the final aldolase structure appears as expected for a high-quality cryo-EM structure (Figure 5C), where the phase randomization showed expected low resolution (unlike prior FSC curves Figures 2F & 3D). This structure demonstrates that computational correction of microscope aberrations and particle motion allows for sub-3Å structure determination.

**DISCUSSION**

**Single particle analysis of aldolase with significant microscope aberrations**

The dataset analyzed in this work represents a near worse-case scenario for microscope aberrations. Namely, through the use of beam-image shift data collection in combination with a Talos Arctica that was not previously aligned, there were significant microscope aberrations introduced into the raw data. These aberrations were significant enough to cause objective astigmatism in micrographs due to a large amount of beam tilt (Figure 2A, right).

Despite the presence of significant aberrations, analysis of resulting aldolase particle stacks allowed for 2D and 3D averaging. The 2D class averages obtained from RELION for aldolase (Figure 2C) are indistinguishable from previous published aldolase class averages (Herzik et al., 2017; Kim et al., 2018), indicating that the aberrations do not affect 7-10Å-resolution class averages. Importantly, however, 3D refinement of the original particle stack does not achieve better than 5.6Å resolution (Figure 2E), which is much lower than typical aldolase reconstructions that are within the range of 3-4Å for initial 3D refinements (Herzik et al., 2017; Kim et al., 2018). This analysis indicates that microscope aberrations do not affect sample screening and initial 2D averaging, however, the aberrations prevent structure determination <5Å and may introduce artifacts that affect FSC calculation.

**Significant improvement of resolution through iterative beam tilt correction**

By taking advantage of microscope aberration correction in RELION-3.1 (Zivanov et al.; Wu et al.) we were able to improve the resolution of aldolase from 5.6Å to 2.8Å. While previous work demonstrated that aberration refinement allows
for resolution improvements for data at both 300 keV (Zivanov et al., 2018) and 200 keV (Zivanov et al.; Wu et al.), all datasets analyzed were collected on relatively well-aligned instruments. With high quality starting data, the initial reconstructions prior to aberration correction

Figure 4 - Iterative CTF-refinement with particle polishing improves overall resolution to 2.8Å. (A) Initial 3D structure at 5.6Å. Following the first CTF refinement and 3D refinement to obtain a structure at 4.2Å (B), continued CTF refinements alongside Bayesian particle polishing allowed for resolution and B-factor improvements (C-I), ultimately allowing the determination of a 2.8Å structure (I).

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achieved ~3Å. Moreover, the data collected at 200 keV (Wu et al.; Herzik et al., 2017) used stage position instead of beam-image shift, further minimizing microscope aberrations in the dataset.

Using these algorithmic improvements (Zivanov et al., 2018) in combination with Bayesian particle polishing (Zivanov et al., 2019), we were able to improve the resolution of aldolase to 2.8Å (Figure 4). Analysis of the measured beam tilts indicate that there was beam tilt on the microscope, as seen by the location of 0 mrad beam tilt located off axis from the position of [0,0] beam-image shift (Figure 3E). This confirms that the microscope was not aligned prior to data collection, where well-aligned instruments should have minimal beam tilt when no beam-image shift is applied. In this example, there was significant beam tilt in both X (-1.19 mrad) and Y (1.07 mrad) directions.

Despite utilizing microscope aberration correction and particle polishing, the per-particle data quality remains worse than stage position-collected aldolase data. By comparing the final B-Factor from our data collected using beam-image shift (-55Å²) with aldolase determined from stage position (-35Å²) (Herzik et al., 2017), the higher B-Factor for our data indicates that per-particle signal is lower for our dataset. We do not know if alternative data processing strategies are needed for beam-image shift data collection or whether our sample preparation of aldolase is of poorer quality, but further work is needed to verify if beam-image shift B-Factors are consistently higher than stage position collected data at 200 keV.

**Data throughput vs. data quality**

The main motivation to utilize beam-image shift for data collection instead of stage position is the increased data collection throughput. For the dataset collected here, we were able to obtain a 2.4X increase in throughput for beam-image shift when compared with stage position: 73 movies per hour (beam-image shift) vs. 30 movies per hour (stage position). Considering the cost of instrument time, beam-image shift provides 1,752 movies per 24 hour period vs. 720 movies per 24 hour period for stage position. Indeed, the latest generation of detectors that have faster readout stand to triple this throughput for beam-image shift.

Based on our analysis of aldolase, we believe that there is a significant difference between 200 keV vs. 300 keV beam-image shift data collection (for instances where there is not an optical correction...
on the microscope). At 300 keV, it is possible to use comparable beam-image shift as that used in this study but instead obtain a structure ~3Å (Zivanov et al., 2018). For this dataset at 300 keV, beam-image shift provide high-resolution structures prior to aberration correction. Unlike this previous study, the aldolase structure collected using beam-image shift at 200 keV was limited in resolution due to aberrations to 5.6Å. In order to correct for the aberrations, significant effort was required in order to perform optical grouping and analysis, steps that may be beyond beginning to intermediate RELION users.

With these considerations, we advocate beam-image shift shift at 200 keV for sample screening. This is because we observed high-quality 2D class averages for aldolase despite significant beam tilt, information well-suited for sample screening (i.e. changing buffers, sample concentrations, etc.). However, this study does indicate that even if a user collected data with significant beam tilt from beam-image shift data, software-based aberration correction is possible to <3Å for well-behaved samples like aldolase.

**DATA ACCESSIBILITY**

Cryo-EM structures have been deposited to the EMDB under accession codes EMDXXXX, .... All movies, micrographs, particle stacks, and metadata files are deposited to EMPIAR under XXXX.

**ACKNOWLEDGEMENTS**

We would like to thank all members of the cryo-EM community at the University of Michigan. We would like to particularly thank Dr. Min Su for stimulating discussions related to beam tilt and microscope aberration correction. This work was supported by NSF-DBI-ABI 1759826 (Y.L. & M.A.C.). The research reported in this publication was supported by the NIH under award number S10OD020011.

**METHODS**

**Sample preparation.** Pure aldolase isolated from rabbit muscle was purchased as a lyophilized powder (Sigma Aldrich) and solubilized in 20 mM HEPES (pH 7.5), 50 mM NaCl at 1.6 mg/ml. Sample as dispensed on freshly plasma cleaned UltrAuFoil R1.2/1.3 300-mesh grids (Electron Microscopy Services) and applied to grid in the chamber of a Vitrobot (Thermo Fisher) at ~95% relative humidity, 4°C. Sample was blotted for 4 seconds with Whatman No. #1 filter paper immediately prior to plunge freezing in liquid ethane cooled by liquid nitrogen.

**Cryo-EM data acquisition and image processing.** Data were acquired using the Leginon automated data-acquisition program (Suloway et al., 2005). Image pre-processing (frame alignment with MotionCor2 (Zheng et al., 2017) and CTF estimation using CTFFIND4 (Rohou & Grigorieff, 2015)) were done using the Appion processing environment (Lander et al., 2009) for real-time feedback during data collection. Images were collected on a Talos Arctica transmission electron microscope (Thermo Fisher) operating at 200 keV with a gun lens of 6, a spot size of 6, 70 μm C2 aperture and 100 μm objective aperture using beam-image shift. Movies were collected using a K2 direct electron detector (Gatan Inc.) operating in counting mode at 45,000x corresponding to a physical pixel size of 0.91 Å/pixel with a 10 sec exposure using 200 ms per frame. Using an exposure rate of 4.204 e/pix/sec, each movie had a total dose of approximately 42 e/A² for the 2,111 movies over a defocus 0.8-2 μm.

**Pre-processing.** Movies were aligned using RELION-3.0 (Zivanov et al., 2018) (3.0-beta-2) motion correction with 5 patches in both X & Y directions, a B-Factor of 150Å² without binning. Following motion correction, CTF estimation was performed with CTFFIND4 (Rohou & Grigorieff, 2015) using exhaustive search for a defocus range of 0.5 to 5.0 μm (0.05 μm step size) and an astigmatism search range of 0.5 μm within a resolution range of 6 and 30Å. The combination of
a large astigmatism search with exhaustive searches led to many over-estimates of CTF resolution fits for this dataset. Therefore, in order to remove micrographs automatically, we utilized our recently developed MicAssess (Li et al.) program to remove all empty and bad micrographs. This removed 685 micrographs, leaving 1,426 micrographs for particle picking. Particles were picked from aligned micrographs using crYLO (Wagner et al., 2019) general model PhosaurusNet with an anchor size of 98 x 98 pixels.

**Single-particle analysis without aberration correction.** For 2D classification, 718,578 particles were extracted with an unbinned box size of 300 pixels and subsequently binned to 2.73Å (box size 100 pixels). Particles were then subjected to 2D classification into 100 classes using RELION-3.0.2 (T=2; Iter=25). After selecting particles from the best classes, 275,487 particles underwent 3D classification into 5 classes using RELION-3.0.2 (T=4; Iter=25) and EMD-8743 (Herzik et al., 2017) as a reference model. Following the selection of the best classes, 186,841 particles were centered and re-extracted at 0.91Å/pixel. This stack was used for 3D refinement to obtain a post-processed structure with a resolution of 5.6Å and a B-Factor of -231Å².

**Aberration correction and particle polishing.** Particles were grouped into optics groups based on beam-image shift values obtained from the Leginon database. In order to group particles into discrete optics groups, the entire file of beam-image shift values were divided into 2x2, 5x5, 10x10, or 20x20 evenly spaced groups. The first two beam tilt estimation steps (CtfRefine #1 & #2, Figure 4) used RELION-3.0 (3.0-beta-2). Subsequent steps (Polish #1 & #2, CtfRefine #3, #4, #5, & #6) used RELION-3.1 (version 30001). All steps for aberration correction and polishing are described in Figure 4. Aberration correction and polishing did not improve resolution more than the final 2.8Å aldolase structure.

**Model building and refinement.** The coordinates for rabbit aldolase (PDB: 5vy5) were docked into each map in PHENIX using phenix.dock_in_map (Adams et al., 2012). Structure refinement and model validation were performed using phenix.real_space_refine (Afonine et al., 2018). The same docking and refinement parameters were used for each map. To make figures showing map density, phenix.map_box was used to restrict the map shown to specific stretches of residues. Root mean square deviation (rmsd) values comparing all atoms between structures were calculated using a Least Squares Fit in Coot (Emsley et al., 2010). The PyMOL Molecular Graphics System (Version 2.1, Schrödinger, LLC) was used to render images showing these structures.

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Supplemental Figure 1 - Representative densities from iterative beam-tilt refinements. Sharpened densities with associated models highlight changes in density quality through iterative rounds of beam-tilt refinement.
Supplemental Table 1 - Cryo-EM data collection, refinement and validation statistics.

|                          | Aldolase (19apr12a) |
|--------------------------|---------------------|
| **Microscope**           | Talos Arctica       |
| **Detector**             | Gatan K2            |
| **Voltage (kV)**         | 200                 |
| **Electron exposure (e^-/Å²)** | 43                 |
| **Defocus range (μm)**   | 0.8 - 2             |
| **Data collection mode** | Beam-image shift    |
| **Micrographs collected (per hour)** | 73             |
| **Original pixel size (Å)** | 0.91             |
| **Symmetry imposed**     | D2                  |
| **Number of micrographs**| 2,111               |
| **Initial particle images (no.)** | 718,578             |
| **Final pixel size**     | 0.91                |
| **Final particle images (no.)** | 186,841            |
| **Number of optics groups** | 400             |
| **FSC threshold**        | 0.143               |
| **Final map resolution (Å)** | 2.8            |
| **Final B-Factor (Å²)**  | -55                 |
Supplemental Table 2 - Model building statistics.

| Structure from Figure 4 | 4A  | 4B  | 4C  | 4D  | 4E  | 4F  | 4G  | 4H  | 4I  |
|-------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Resolution (Å)          | 5.6 | 4.2 | 3.8 | 3.3 | 3.3 | 3.0 | 3.0 | 2.9 | 2.8 |
| B-Factor (Å²)           | -237| -214| -164| -129| -111| -93 | -67 | -65 | -55 |
| Bonds (RMSD)            |     |     |     |     |     |     |     |     |     |
| Length (Å)              | 0.005| 0.005| 0.005| 0.005| 0.005| 0.006| 0.005| 0.005| 0.005|
| Angles (°)              | 1.049| 0.871| 0.834| 0.830| 0.764| 0.933| 0.862| 0.838| 0.875|
| Molprobity score        | 2.28 | 1.83 | 1.72 | 1.67 | 1.43 | 1.81 | 1.78 | 1.5 | 1.60 |
| Clash score             | 10.54| 3.70 | 3.23 | 4.08 | 4.18 | 3.32 | 3.04 | 3.09| 3.09 |
| Ramachandran plot (%)   |     |     |     |     |     |     |     |     |     |
| Outliers                | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| Allowed                 | 3.52| 2.93| 3.81| 3.45| 3.52| 4.33| 3.81| 4.11| 4.40|
| Favored                 | 96.48| 97.07| 96.19| 96.55| 96.48| 95.67| 96.19| 95.89| 95.60|
| Rotamer outliers (%)    | 4.68| 4.68| 2.88| 2.16| 0.36| 3.24| 3.60| 1.44| 1.80|
| CaBLAM outliers (%)     | 2.36| 3.17| 2.58| 2.65| 2.36| 2.95| 2.95| 2.65| 2.36|
| CC (mask)               | 0.79| 0.84| 0.82| 0.82| 0.82| 0.81| 0.81| 0.81| 0.81|
| CC (box)                | 0.81| 0.80| 0.83| 0.76| 0.77| 0.75| 0.74| 0.75| 0.76|
| CC (peaks)              | 0.72| 0.75| 0.77| 0.72| 0.73| 0.71| 0.71| 0.72| 0.73|
| CC (volume)             | 0.80| 0.83| 0.81| 0.81| 0.81| 0.80| 0.79| 0.80| 0.80|
| RMSD (Å) all atoms      | 1.02| 0.82| 0.81| 0.63| 0.57| 0.53| 0.54| 0.49| -   |
| compared to Figure 4I   |     |     |     |     |     |     |     |     |     |