THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Comparison of CryoSPARC and RELION in Cryo-EM Single Particle Analysis

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ABSTRACT

Since the development of cryo-electron microscopy (cryo-EM), a new tool for structural biologists emerged that eliminated some of the challenges presented by other techniques, such as X-ray crystallography. For example, the algorithms in the 3-D reconstructions using cryo-EM demonstrate the capability of providing structural information with tolerance to sample heterogeneity. Structure determination by single-particle cryo-EM has since become instrumental in the fields of virology, biochemistry, and progression in drug discovery pipelines. Single-particle analysis of cryo-EM data is facilitated through software program tools that are optimized to configure single-particle reconstructions. Among the frontiers of these software programs are cryoSPARC and RELION that operate in the Bayesian likelihood framework. Additionally, the framework surrounding these software programs is supported and further analyzed by an interactive visualization and analysis program called UCSF Chimera.

CryoSPARC and RELION integrate similar job functions in their workflow, including but not limited to 2D classification, *ab initio* 3D classification, and CTF refinement. While the workflow of single-particle reconstruction refined by cryoSPARC and RELION has similarities, the framework behind these software tools has differences in the algorithm. We investigated the two software programs, cryoSPARC and RELION, in a comparative study to expand on the similarities and further elucidate the differences. The objective of this comparative study is to inspect the parameters utilized by the two software programs for jobs to evaluate the implications on the resolution of specimens.

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ACKNOWLEDGEMENTS

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Chapter 1 Introduction

Development of Cryogenic Electron Microscopy (cryo-EM)

The technological advancements in electron microscopy unveiled the unprecedented capabilities of producing high-resolution images of biological specimens by imaging devices that use electrons (1). Comparably to its X-ray crystallography and NMR counterparts, the emergence of cryo-EM as a mainstream technique in structural determination resulted from addressing fundamental questions. Dubbed the "Resolution Revolution," these fundamental questions were addressed by scientists—Jacques Dubochet, and Richard Henderson, and Joachim Frank —who made the greatest contribution to three critical stages of cryo-EM: sample preparation, data acquisition, and 3-D reconstruction, respectively (1). The combined efforts for the development of these three critical stages of cryo-EM were recognized and the three scientists were awarded the Nobel prize in 2017. The assembly of randomly orientated biomolecules in solution suspended in a thin film of vitreous ice, through a technique called vitrification, governs a branch of cryogenic electron microscopy designated single-particle analysis (2). The preservation of biomolecules in vitreous ice is proceeded by the collection of thousands of micrographs that are processed by scientific software platforms. Such software programs, such as cryoSPARC and RELION, utilize specific methodologies to reach a near-atomic resolution of the biomolecule (3). From helical reconstructions of filamentous amyloid fibrils to elucidating the unique architecture of icosahedral viruses, single-particle cryo-EM has pushed past barriers that were formerly difficult to surpass (1-3). Structural determination of biological specimens, in particular macromolecules, relies heavily on specimen isolation and

purification in order to compute complex 3-D structures and enhance the definition of local environments (4). The methodologies for structure determination by single-particle analysis by cryo-EM follow a specific protocol (Fig. 1).

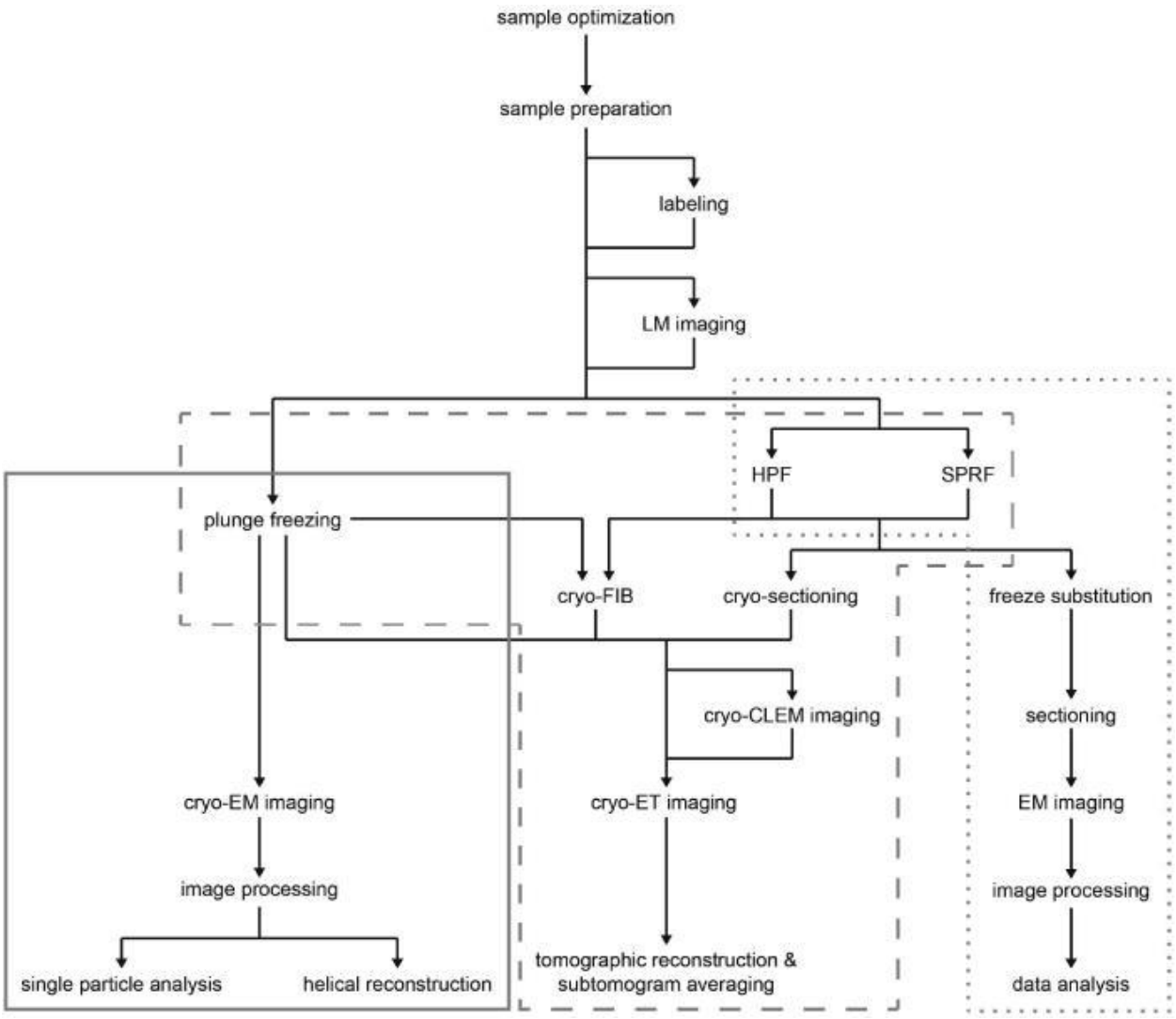

Figure 1. A schematic illustration of the workflow options adapted for cryo-EM.

A visual representation of the workflow options for cryo-EM reconstructions. This is schematic illustration is adapted by Dillard et al. 2018 (6) and presents a comprehensive examination of options for the three phases of cryo-EM noted earlier: sample preparation, data acquisition, and 3-D reconstruction.

The grey solid box depicts the illustration a branch of the workflow that leads to single particle analysis (6).

The downstream applications of single particle analysis subject the captured images of macromolecules to computational analysis.

Single Particle Analysis

Sample Heterogeneity

Several limitations imposed by X-ray crystallography on structural determination are primarily due to intolerance to sample heterogeneity that prevents the solution of the structure (5). One of the challenges presented by sample purification is the preservation of structural homogeneity that may interfere with structural determination (6). While purified macromolecular specimens should conserve some range of homogeneity, cryo-EM adapted to conformational and compositional variability in samples by incorporating techniques and algorithms in the computational analysis (4,6,7).

Specimen Preparation and Downstream Applications

Before data collection and subsequent imaging analysis, the expression of the specimen of interest and its structural integrity are verified through biochemical methods (4,7). Imaging for single-particle cryo-EM, as indicated in Fig. 1, requires directing the specimen for electron microscopy to be mediated through EM grids that provides support, typically a carbon film with

a support structure (4,8). The examination of specimens for single particle cryo-EM subjects EM grids to one of two methods for data analysis: (1) Negative staining, (2) Vitrification. As indicated on the right arm of the branch of Fig. 2, specimens embedded in vitreous ice with different orientations permit further analysis beyond 2D averaging (7). Vitrified specimens in EM grids that have achieved high density and different orientations are excellent candidates for 3-D reconstructions (4,6,7,8). The continuation of 2D averages to imaging process by software programs permits sample refinement that yields a higher resolution than the EM negative staining (7,8).

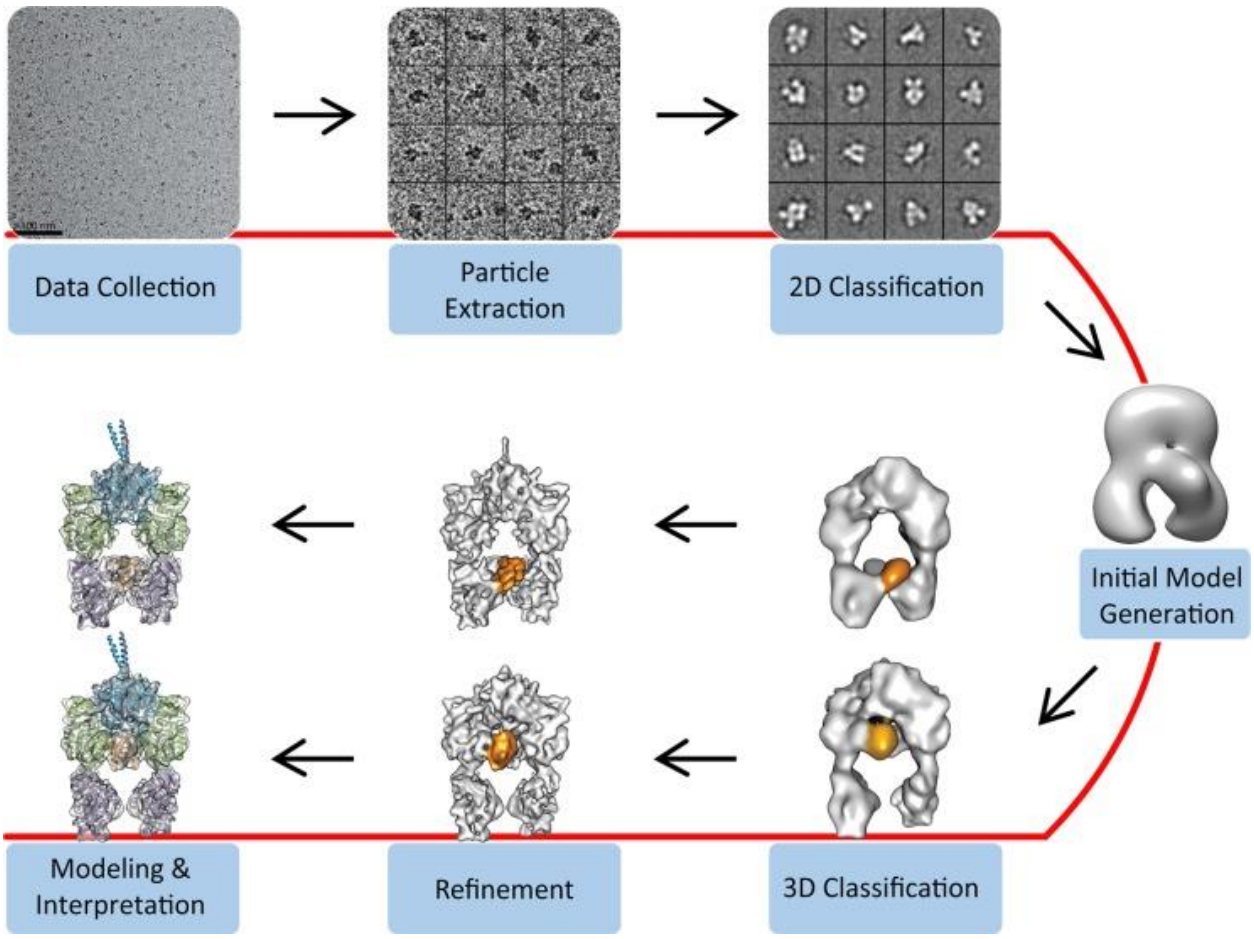

Figure 2. An illustration of the workflow for structure determination by single-particle cryo-EM.

A workflow of the steps involved in single-particle cryo-EM by two methods: negative staining and vitrification. Negative staining is a well-established technique used in electron microscopy with variations to perfect 2D classification (9). Cryo-EM single particle analysis uses a vitrification technique that enables 3-D reconstructions and modeling downstream via software programs. This workflow is adapted by Skiniotis and Southworth, 2015.

Previously described as a bottleneck, the data collection automated by electron microscope has significantly advanced by reducing the time to collect the data (10). However, the technology for 3-D reconstructions efficacy did not reach its full capacity (10). Here, we inspect two software platforms, cryoSPARC and RELION, that are adapted to configure 3-D reconstructions by single-particle analysis from cryo-EM data. These two software programs, among others, challenge this bottleneck to compress the time required for 3-D reconstructions.

Cryo-SPARC

CryoSPARC, or Cryo-EM Single Particle Ab-Initio Reconstruction and Classification, is a software program adapted for solving cryo-EM structures of viruses, membrane proteins, in addition to negative staining data. This software program for single-particle analysis integrates graphics processing unit (GPU) as a package to deliver high-resolution refinements of 3-D structures obtained from cryo-EM data (10). The development of cryo-SPARC aims to enable a user-friendly interface for structural determination of single-particle images by incorporating a

stochastic gradient descent (SGD) to the maximum likelihood optimization algorithm (10). The computational analysis behind this software is dependent on two algorithms that are refined to expedite the 3-D reconstructions: (1) unsupervised ab initio 3-D classification and (2) removal of redundant computation to enhance the high-resolution refinement of cryo-EM maps (10). As dictated in Fig. 2, the 3-D reconstruction follows a series of jobs that are dependent on the parameters as a guide to achieving high-resolution reconstructions.

RELION

An alternative to cryo-SPARC for obtaining macromolecular structures by refinement also uses the Bayesian likelihood framework but utilizes a different approach for 3-D reconstructions (11-12). RELION, which corresponds to REgularized LIkelihood OptimizatioN, replaces the manual application of adjusting parameters by the user to an inferential decision of parameters dictated by the Bayesian approach (12). As described as a potential pitfall for new users, identifying and inserting parameters for jobs in the steps of computing the 3-D reconstructions may interfere with the refinement process and lead to erroneous structures (11,12). The algorithms used by RELION attempts to overcome this potential pitfall by using the Bayesian approach.

One of the common problems encountered by structural determination by cryo-EM is overfitting and over-estimation of resolution (13). A method to prevent the overfitting and over-estimation of the resolution, thus preventing inaccuracies in the structure, is the application of the gold-standard approach (12-14). In the gold-standard approach, the accuracy of the resolution obtained by the refinement is validated by two independent reconstructions that provide the

Fourier shell correlation (FSC) for both reconstructions (13). This approach and the utility of accurate resolution determination were implemented in RELION for improvements in reliability (13,14).

Coxsackievirus B3 Introduction and Importance

Coxsackievirus B3, or CVB3, belongs to the *Picornaviridae* family and resembles the members of the *Enterovirus* genus that are cardiotropic, indicated by their pathogenic abilities to cause viral myocarditis (15,16). Moreover, the consequence of CVB3-induced viral myocarditis can result in life-threatening disease such as myocardial fibrosis, with a common onset of heart failure (15). Enteroviruses have also been implicated in the development of Type 1 diabetes, with evidence to support increased susceptibility by CVB3 infections (17). CVB3 are small nonenveloped viruses that are roughly ~30 nm in diameter in which a positive sense (+) single-stranded RNA genome is contained. The CVB3 virion consists of the assembly of 12 pentamers that constitute an icosahedral capsid structure (15,16). The structural units of a pentamer are five protomers, in which each are composed of four proteins: V1, V2, V3, and V4 (15,16).

The entry of CVB3 into cells is dictated by the presence of a main receptor and coreceptor that establishes cardiomyocytes as their cellular tropism. The main receptor, called coxsackievirus and adenovirus receptor (CAR), coupled with the coreceptor, decay-accelerating factor (DAF), also known as CD55, permit the targeting of cardiomyocytes to facilitate its entry (15,16,18). However, a new variant of CVB3, called CVB3/28, is relieved of the two cellular receptor prerequisites for entry by escaping the requirement of DAF binding (18).

Chapter 2 Materials and Methods

β-galactosidase Reconstruction Protocol

RELION

The tutorial data set on β-galactosidase was kindly provided by Takayuki Kato from the Namba group from Osaka University, Japan. The data collection for this tutorial on β-galactosidase was performed on a 200kV JEOL cryo-ARM microscope. This data set is available on EMPIAR, designated as EMPIAR-10204. The tutorial test dataset was downloaded into a single directory for the insertion of the raw micrographs, the reconstruction of β-galactosidase was completed by the jobs as indicated in Table 1. The local resolution for β-galactosidase was visualized in UCSF Chimera.

Table 1. The jobs performed in RELION for reconstruction.

RELION	
Order of Iterations	**Job**
1	Import
2	Motion correction
3	CTF estimation
4	Auto-picking
5	Particle extraction
6	Subset selection
7	2D classification
8	3D classification
9	3D auto refine
10	CTF Movie refinement

11	Particle polishing
12	Mask creation
13	Join star files
14	Particle subtraction
15	Local resolution

CryoSPARC

The tutorial test dataset was downloaded into the directory in CryoSPARC in preparation for processing for reconstruction. The local resolution for β-galactosidase was visualized in UCSF Chimera.

CVB-28 Reconstruction Protocol

Virus growth and purification

The preparation of the specimen required the propagation of CVB3 strain 28 using cell culturing techniques applied to HeLa cells, as previously described in Organtini et al. 2014 (19). After propagation of CVB3-28, the cells were subjected to a freezing and thawing cycle three times before separation. The viruses were collected by pellets achieved by passing the clarified lysate through a sucrose cushion. Furthermore, the purification was accomplished by using a tartrate step gradient ultracentrifugation.

Vitrification for data collection by cryo-EM

In preparation for collecting electron micrograph, a 2/1 Quantifoil grid with the specimen was assembled by applying a small aliquot. Once the EM grids were prepared, they were vitrified in liquid ethane using a freezing robot.

Data collection by Krios microscope

Electron micrographs were collected on the Krios microscope by recording the images with a Falcon 3 direct electron detector that provided a final pixel size of 1.137 Angstroms.

Chapter 3 Results and Discussion

β-Galactosidase Reconstruction

The β-galactosidase tutorial dataset provided the EM movies for processing in cryoSPARC and RELION. The EM movies were imported into the software programs and parameters were entered to initiate the steps for reconstruction.

CryoSPARC

The EM movies were accessed and entered using /*.tiff to insert the movies into the job function. The following parameters were input to carry out the job.

Table 2. The input for job parameters in cryoSPARC for the reconstruction of β-galactosidase.

Job Parameters	
Raw pixel size (Å)	0.885
Accelerating voltage (kV)	200
Spherical aberration (mm)	1.4
Total expose dose (e/A^2)	49

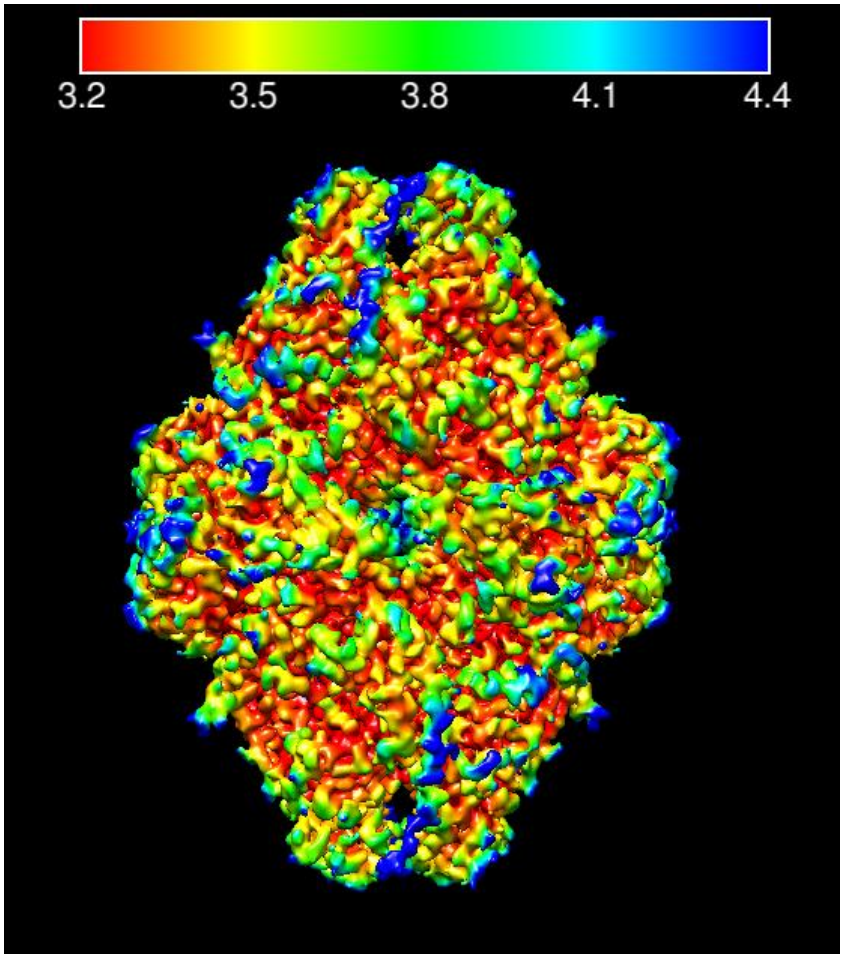

Figure 3. A local resolution of β-galactosidase estimated by cryo-SPARC and visualized using UCSF Chimera.

The completion of the job by cryoSPARC was proceeded with visualization of the local resolution output of β-galactosidase by UCSF Chimera. The values of the resolution range are generated to represent the 3-D colored map in Chimera.

RELION

The EM movies of the β-galactosidase were accessed and inserted for processing using RELION. The parameters are noted in Table 3.

Table 3. The input for job parameters in RELION for the reconstruction of β-galactosidase.

Job parameters	
Raw pixel size (Å)	0.885
Voltage	200
Dose per frame (a/A^2)	1.277
Pre-exposure (e/A^2)	0

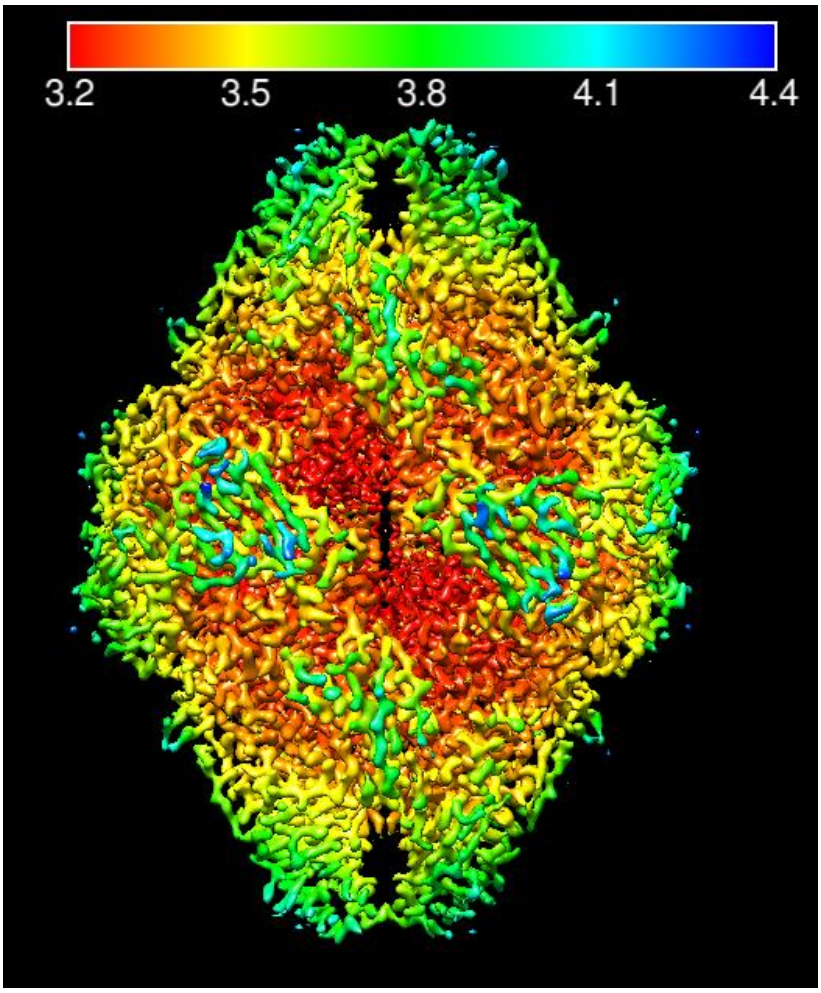

Figure 4. A local resolution of β-galactosidase estimated by RELION and visualized using UCSF Chimera.

The final job, which is the local resolution output, was visualized in UCSF Chimera to illustrate the resolution range.

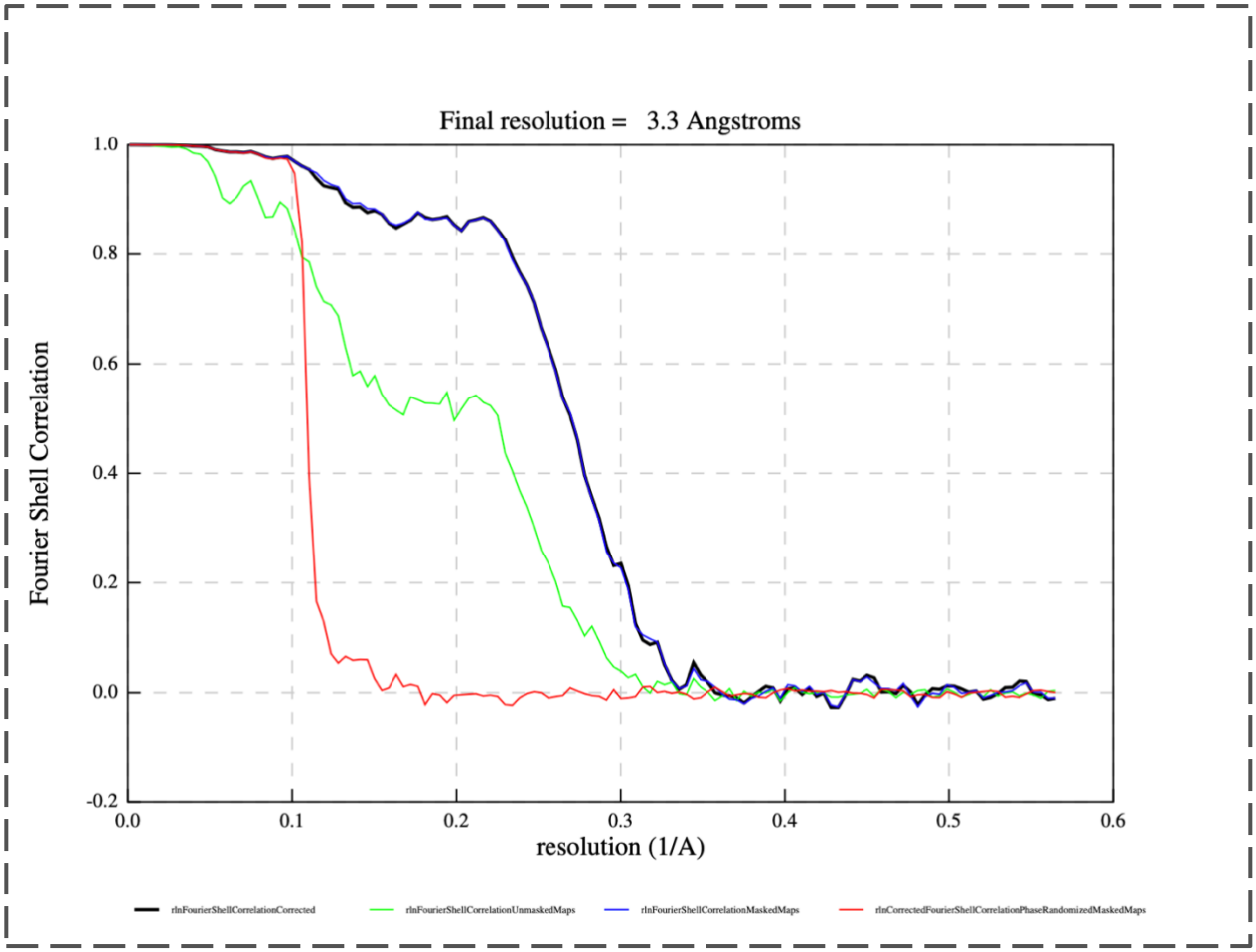

Figure 5. The Fourier shell correlation (FSC) curve for the β-galactosidase reconstruction for refinement assessment.

As represented in Fig. 5, the final resolution was obtained at 3.3 Angstroms from refinement in RELION. The 3-D density map in Fig. 4 is displayed and corresponds to the calculation of the FSC curves as depicted in Fig. 4.

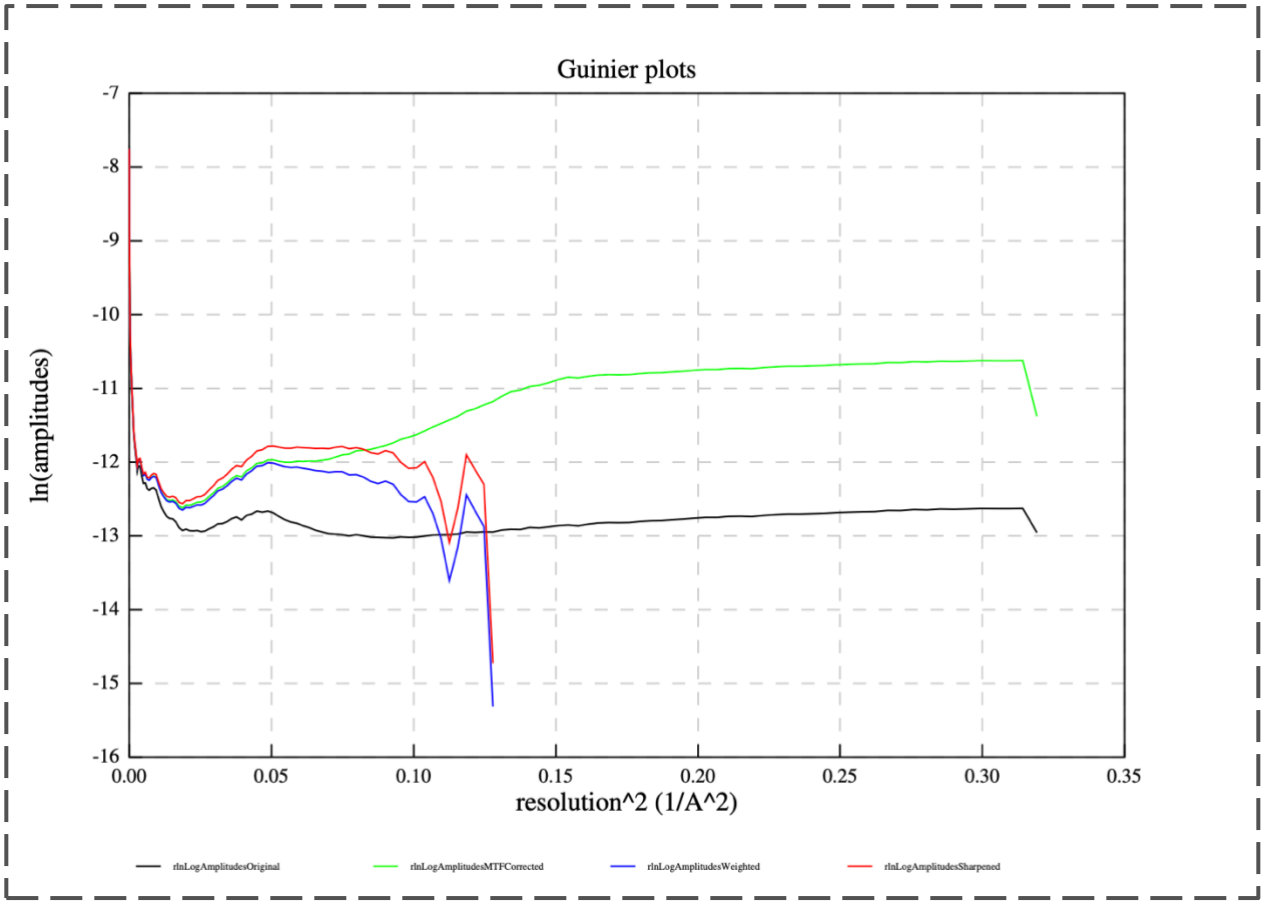

Figure 6. A Guinier plot for β-galactosidase reconstruction.

Full CVB3-28 Virus Reconstruction

The local resolution of CVB3-28 was visualized using Chimera from the outputs of cryoSPARC and RELION. The local resolution jobs were completed by both programs to achieve the density map displayed in Chimera. Following the completion of the jobs, the final resolution for RELION is 3.7 Angstroms whereas cryoSPARC accomplished a reconstruction with a resolution at 3.3 Angstroms. Additionally, the B-factors were recorded from cryoSPARC and RELION and are 127 $Å^2$and 109 $Å^2$, respectively.

CryoSPARC

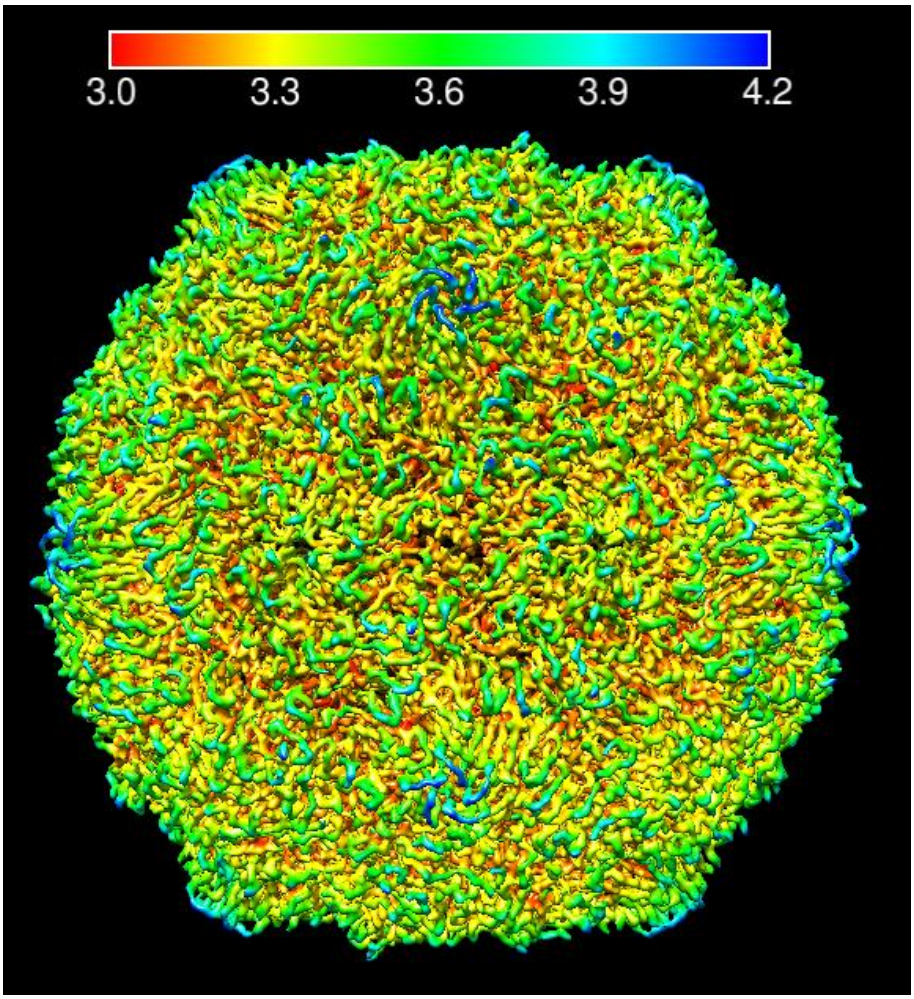

Figure 7. A local resolution of CVB3-28 estimated by cryo-SPARC and visualized using UCSF Chimera.

RELION

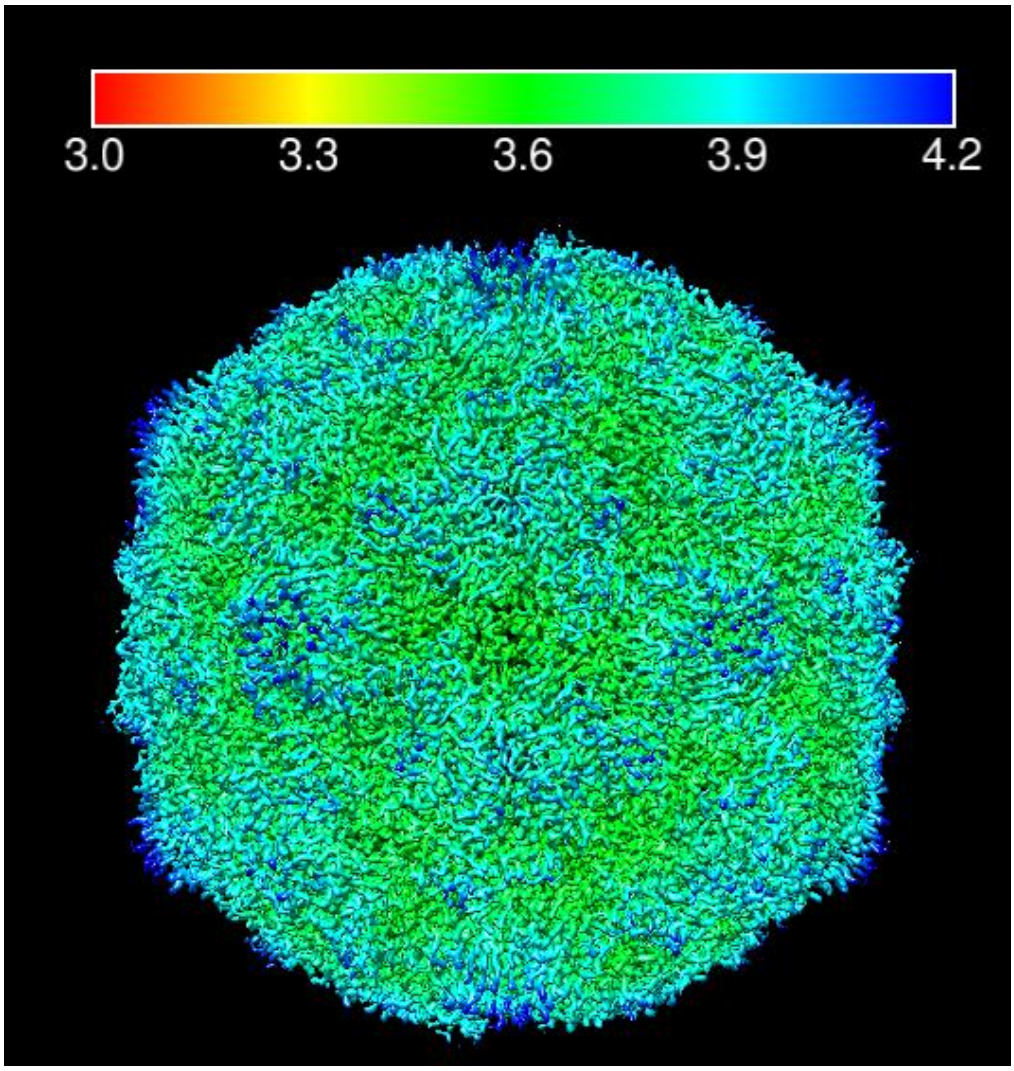

Figure 8. A local resolution of CVB3-28 estimated by RELION and visualized using UCSF Chimera.

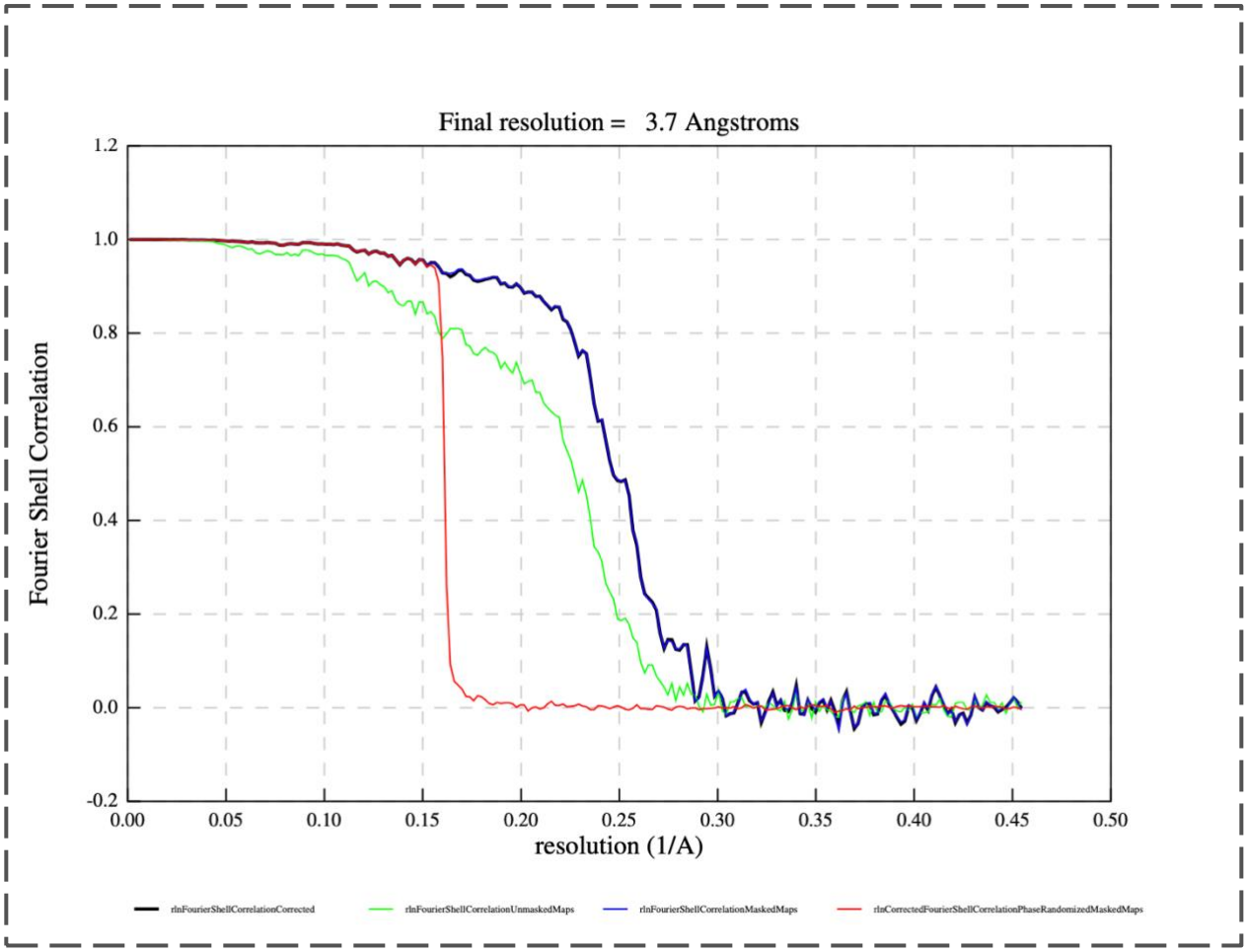

Figure 9. The Fourier shell correlation (FSC) curve for the CVB3-28 reconstruction for refinement assessment.

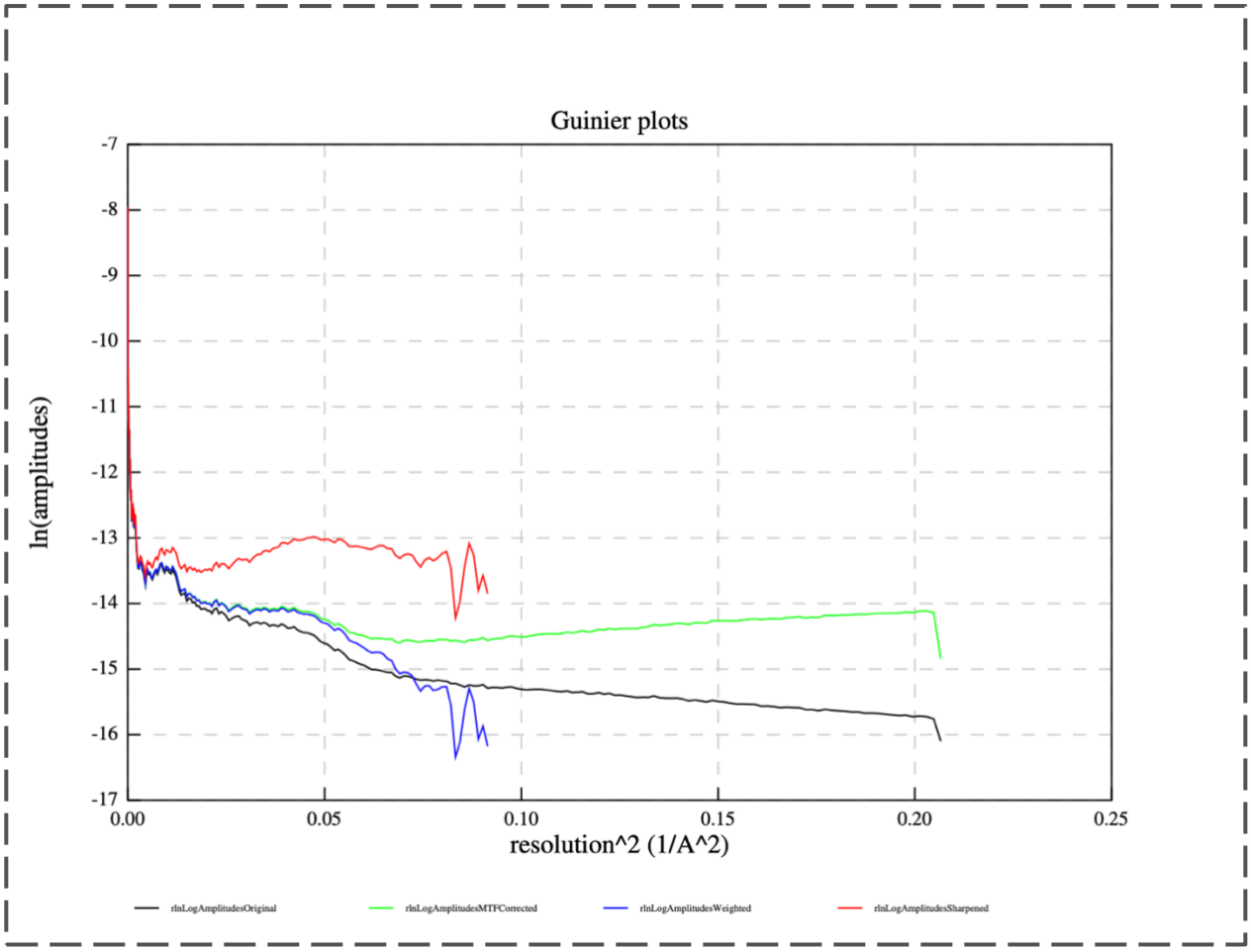

Figure 10. A Guinier plot for CVB3-28 reconstruction.

Chapter 4 Conclusion

Summary

Investigating potential pitfalls in computer software programs that reconstruct macromolecules by single particle analysis could ameliorate the strategies and accuracy of obtaining 3-D macromolecular reconstructions. In this study, we proposed a comparative analysis of cryoSPARC and RELION in their refinement strategies to reconstruct 3-D macromolecular structures. One of the known erroneous causes of inconsistences in structural determination is the overfitting of data (14). RELION overcomes this hurdle by implementing algorithms in the gold-standard approach. Two independent 3-D reconstructions are processed to validate the final resolution using the FSC curves (14). The FSC curves were generated for refinement processed by RELION as indicated in Figures 5 and 9. The two independent refinements in the gold-standard FSC may eliminate any biases in the 3-D reconstruction by RELION that may be present in cryoSPARC. Furthermore, the gold-standard FSC provides a more representational determination of structures (20). This approach is also reliable in reproducing consistent 3-D maps in downstream applications for the purposes of modeling and interpretation of the macromolecular structure, also noted by Natesh (2019). The FSC curves generated for the reconstructions of the β-galactosidase and CVB3/28 by RELION provides a useful resource in which the preventive measures against over-fitting accurately ensure validity of structure and final resolution.

The modeling and interpretation of the macromolecular structures at high-resolution can be greatly optimized by implementing approaches that minimize the loss of contrast (21). By implementing these strategies, high-resolution of the specimen can be captured to deduce

structural features and information at high-resolution. Contrast loss can be depicted in Guinier plots by extrapolating the B-factors from the plot. The B-factors for the CVB3/28 reconstructions on cryoSPARC and RELION are 127 $Å^2$and 109 $Å^2$, respectively. The Guinier plots were imaged from the software for analysis. In contrast to the 3-D reconstruction of β-galactosidase and the final resolutions obtained, cryoSPARC generated a higher resolution reconstruction of CVB3/28 at 3.3 Angstroms compared to 3.7 Angstroms generated by RELION.

Future Direction

Several approaches have been proposed to eliminate sources of errors, such as heterogeneity, in the 3-D reconstruction process to generate macromolecular structure that are high in resolution for easy accurate interpretation (21). One future direction for improvement of cryo-EM single particle analysis is refining the algorithm to tolerate high degrees of heterogeneity.

Appendix A

Version of software programs used for 3D-reconstructions.

CryoSPARC: Version 3.0

RELION: Version 3.1

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ACADEMIC VITA

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EDUCATION

BS The Pennsylvania State University-University Park — Fall 2017-present
Schreyer Honors College | Biochemistry and Molecular Biology
Minor in Microbiology
Expected graduation date: May 2021

HONORS AND AWARDS

Eberly College of Science Dean's Scholars Program — Fall 2019-present

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Recognizes academic excellence of full-time students that obtain a semester GPA of 3.50 or higher.

Charles R. Gerth Scholarship — Fall 2020-Spring 2021
Science Dean's Scholarship — Fall 2019-Spring 2020
Irvine and Jeanne Atlas Scholarship in Biochemistry — Fall 2019-Spring 2020

RESEARCH EXPERIENCE

Undergraduate Research — Fall 2019-present
Hafenstein Lab – Department of Biochemistry and Molecular Biology
Thesis title: "Comparison of CryoSPARC and Relion in Cryo-EM Single Particle Analysis"

- Investigating the structure of coxsackievirus B3 (CVB3) in wet lab and computational lab settings.
- Utilize software tools, CryoSPARC and Relion, to address the structure of CVB3.
- Conducted a comparative analysis study to distinguish features in cryo-EM software that contribute to high-resolution structures of molecules.

Summer Research Internship Program — May 2019-Aug 2019
Penn State College of Medicine – Hershey, PA
Mentor: Dr. Shengyu Yang, PhD – Department of Cellular and Molecular Physiology
Thesis title: "Mitochondrial Calcium Uniporter Overexpression Sensitizes Pancreatic Ductal Adenocarcinoma Cells to Ferroptosis"

- Research in investigating the ferroptosis pathway in cancer metastasis and cancer metabolism.
- Conducted a series of experiments focused on observing the effects of chemotherapy drugs on pancreatic ductal adenocarcinoma (PDAC) cell line.
- Use of academic resources provided by the program, including scientific research seminars, career development, and workshops.

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- Utilizing bioinformatics and genomic software such as DNA Master to annotate a phage virus genome.

WORK EXPERIENCE

Clinical Information Specialist (Medical Scribe) | CIMS — Nov 2020-Present

Utilized clinical documentation tools (EPIC) for scribing to ease the administrative burdens of physicians and enhance patient interaction.

- Learned medical terminology and mastered type writing in EPIC for both ED and outpatient settings. Maintained and revised medical documentation in EPIC.
- Work in a collaborative atmosphere with a physician to enhance the interaction between the physician and patient.
- Maintained and revised medical documentation in EPIC throughout the entire visit of patients and knowledge in diverse medical visits.

Public Health Ambassador Aug 2020-Nov 2020

Selected as a public health ambassador to maintain safety on campus and to mitigate the spread of SARS-CoV-2.

- Direct the 6-feet distance order to students on campus.
- Hand out bags that contain masks, hand sanitizer, and a card containing safety rules.
- Worked with other public health ambassadors to ensure people are following the new regulations during the COVID-19 pandemic.

Guided Study Group (GSG) leader | Penn State Learning (PSL) Aug 2019-Dec 2019

My position as a GSG leader is to organize sessions for students in a Molecular and Cellular Biology course to attend and engage in group discussions for a deeper comprehension of the material.

- Attend lectures to keep up with the material presented in class
- Created material for every session, including worksheets, PowerPoint slides, group discussion questions, etc.
- Hosted two sessions a week for 1 hour and 30 minutes and 2-hour exam review sessions.
- Completion of a complimentary tutoring course: CI 200: Peer Tutoring.

POSTER PRESENTATION

STEP-UP Annual Undergraduate Scientific Session and Research Presentations at the National Institutes of Health

Bethesda, MD Aug 2019

Title: "Mitochondrial Calcium Uniporter Overexpression Sensitizes Pancreatic Ductal Adenocarcinoma Cells to Ferroptosis"

- Poster and Oral presentations over a span of two days.

VOLUNTEERING/COMMUNITY SERVICE

Patient Ambassador | COVID-19 Hope Squad March 2020-Present

Assisting in the operation of vaccine clinics opening for 3 months across Southern PA. The volunteer program was developed by WellSpan to deliver vaccines to the community.

- Adhered to the COVID-19 protocols for safe and easy accessibility for patients to receive the vaccine.
- Assisted patients to the vaccine clinic and provided support, if necessary, for patients in wheelchairs, etc.
- Greeted patients asked specific questions pertaining to COVID-19 standards.
- Took temperatures and guided the patients to specific locations at the site.

Patient Care Volunteer | VNA Hospice Aug 2020-Present

A hospice volunteer in my community to gain insight into the hospice system and support patients in their final stage of life.

- Adhered to the COVID-19 protocol to maintain safety with the staff and patients.
- Helped and engage the patients in meaningful activities throughout the day.

Hotline Counselor/Advocate | Centre Safe Aug 2020-Present
A hotline counselor/advocate for the Centre County area. Training to assist victims of domestic violence, sexual assault, stalking, etc. 80-hour training program with a practicum completed to prepare volunteers to answer hotline calls.

- Completed the training that prepares us for taking calls from victims of domestic abuse, stalking, etc.
- Knowledge of filing ePFAs, SART process, and other protocols for helping victims.
- Worked as a team with other Hotline counselors/advocates to ensure the victims receive necessary help such as transportation to courthouses, SART call, etc.

CERTIFICATES

Leadership Education and Development (LEAD) Program Spring 2020
Pennsylvania State University-University Park

SKILLS

- DNA Master: bioinformatic software
- Cell culture procedure and technique
- Genome annotation using software programs
- Scientific writing methodology knowledge and practice

LANGUAGES

English: Native Language
Urdu: Native language