



Aug 28, 2024

🌐 Single particle analysis of α -Synuclein fibrils

📖 Molecular Neurodegeneration

DOI

<https://dx.doi.org/10.17504/protocols.io.81wgbxozylpk/v1>

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DOI: <https://dx.doi.org/10.17504/protocols.io.81wgbxozylpk/v1>

External link: <https://doi.org/10.1186/s13024-025-00889-y>

Protocol Citation: Kevin Sicking, Rubén ernández-Busnadiego 2024. Single particle analysis of α -Synuclein fibrils. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.81wgbxozylpk/v1>

Manuscript citation:

Brücke C, Al-Azzani M, Ramalingam N, Ramón M, Sousa RL, Buratti F, Zech M, Sicking K, Amaral L, Gelpi E, Chandran A, Agarwal A, Chaves SR, Fernández CO, Dettmer U, Lautenschläger J, Zweckstetter M, Busnadiego RF, Zimprich A, Outeiro TF (2025) A novel alpha-synuclein G14R missense variant is associated with atypical neuropathological features. Molecular Neurodegeneration 20(). doi: [10.1186/s13024-025-00889-y](https://doi.org/10.1186/s13024-025-00889-y)

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Protocol status: Working

We use this protocol and it's working

Created: December 29, 2023

Last Modified: August 28, 2024

Protocol Integer ID: 92909

Keywords: ASAPCRN, synuclein fibril, amyloid fibril, synuclein, syn fibril, structural information of these fibril, single particle analysis, specialized single particle analysis, fibril, dementia with lewy body, parkinson, including parkinson, syn

Funders Acknowledgements:

MJFF/ASAP

Grant ID: ASAP-000282

DFG

Grant ID: EXC 2067/1-390729940

Disclaimer

It is assumed the reader is familiar with the operation of the equipment mentioned herein. To avoid hazards, it is highly recommended to seek advice and training from experienced staff if this is not the case.

Abstract

Aggregates of α -Synuclein (α -Syn) have been implicated in the pathogenesis of a spectrum of disorders, including Parkinson's disease, multiple system atrophy, and dementia with Lewy bodies. These aggregates comprise misfolded α -Syn in the form of amyloid fibrils. Structural information of these fibrils is valuable, as it provides insights into potentially harmful interactions facilitated by amyloid fibrils in their native environment. This comprehensive protocol describes the entire procedure for generating structural information of in vitro α -Syn fibrils through a specialized single particle analysis (SPA) approach (see Figure 1).

Attachments



[937-2418.pdf](#)

601KB



Guidelines

Plunging of α -Syn fibrils

The aim is to carefully place α -Syn filaments on cryo-EM grids and quickly freeze them to create a vitrified state in the fibril solution. This prevents the formation of damaging ice crystals that could harm the soluble proteins. It is crucial to keep the plunge-frozen sample close to $-192\text{ }^{\circ}\text{C}$ (liquid nitrogen temperature) to prevent any thawing. This ensures the α -Syn filaments remain structurally intact for accurate cryo-EM analysis.

Cryo-TEM data collection

The goal is to capture images, in the form of electron event representation (EER) frames, of α -Syn fibrils suspended in vitreous ice on cryo-EM grids. It is important to gather images on holes showing abundant α -Syn fibrils, which at the same time do not overlap significantly (though precise particle picking allows for effective data processing even with some overlapping filaments).

Preprocessing

During this step, EER frames undergo motion correction and conversion into MRC files. Subsequent processing steps will exclusively take place in RELION (Scheres, 2012), leveraging its specialized functions tailored for helical structures, even accommodating unique cases like amyloid helices. Thus, preprocessing also includes importing the MRC files into RELION. Additionally, preprocessing involves the correction of the Contrast Transfer Function (CTF) of images, enhancing the signal-to-noise ratio of images.

Particle picking in crYOLO

During this stage, the crYOLO network, a deep-learning neural network specializing in particle picking (Wagner et al., 2019), is trained and subsequently employed to predict filament positions on all micrographs. These predictions are then imported into RELION for particle extraction. This integrated approach ensures efficient and accurate identification of filament positions.

Note

Depending on the size of the dataset, a computer with at least one to two GPUs should be used. Then training the network and predicting filaments should be done in approximately 12 hours.

2D classifications to get a good particle set

From here on, data processing generally follows Lövestam & Scheres, 2022. The goal is to remove trash particles such as carbon edge picks, particles originating from overlapping filaments, and generally low-quality particles in a binned 2D classification. Since particles are extracted with a larger box size for this binned

classification, the resulting class averages can be used to estimate the crossover distance and hence the helical properties of the amyloid helices (Scheres, 2020). Then, following the selection of reasonable classes, an unbinned 2D classification is carried out to identify particles contributing to beta-sheet separated class averages. Those high-quality particles will be used for 3D classifications later down the line.

Note

Again, depending on the size of the dataset, usage of a high-performance computing cluster is advised. Generally, at least four, for large datasets up to 16 GPUs should be used, especially for the unbinned classifications.

3D classification

The goal here is to generate a first 3D map that can be further refined during auto-refinement and various postprocessing steps. This is arguably the most difficult step in the entire workflow. Given the absence of distinct features in amyloid helices, the 3D alignment algorithm has difficulties converging on correct structures. For a more detailed exploration of this issue, refer to Scheres (2020). Consequently, initiating the 3D classification with correct helical parameters for twist and rise is crucial. The rise is fixed at 4.75 Å, but the twist may vary from polymorph to polymorph.

Note

The usage of a high-performance computing cluster is advised. Especially, if helical parameters are unknown and 3D classifications are used in a brute-force manner to narrow them down, then each classification run should have at least four, if possible 16 GPUs. Otherwise, a single iteration can easily take up to 12 hours.

3D Refinement and postprocessing

In this phase, the initial 3D map is refined using RELION's autorefinement process. However, the efficacy of auto refinement hinges on the high quality of the initial map, so only proceed if 3D classification gives reasonable results. Postprocessing plays a pivotal role in enhancing the map by correcting the B-factor. An essential aspect involves the creation and application of a mask to exclude the solvent area. It is worth noting that this is an iterative process where multiple refinement and postprocessing runs can be sequentially linked to further elevate the quality of the density map. Particularly in later iterations, refining helical parameters using the helical search option becomes valuable, and exploring different symmetry options is prudent in cases involving multiple protofilaments.



Note

3D refinements (and especially postprocessing tasks) usually do not need many computational resources, especially, if run with a small but high-quality particle dataset. Four GPUs should be plenty.

Enhancing the quality of the map / Postprocessing

Finally, in this postprocessing step, the quality of the particle set is bolstered through a series of measures. This includes refining the Contrast Transfer Function (CTF) parameters of particles, improving the motion correction of each particle, and selectively filtering out low-quality particles through 3D classifications, utilizing the refined maps as the initial model. These refinements collectively contribute to the overall fidelity and precision of the particle dataset, ensuring a more accurate representation in the final density map.

Materials

Plunging of α -Syn fibrils

- Tris-HCl buffer

	A	B
	Tris-HCl	30 mM
	Buffered to pH 7.5	

- Waterbath sonicator: Bioruptor UCD-200 Sonication System (Diagenode)
- Cryo-EM grids: R2/1, Cu 200 mesh grid (Quantifoil microtools)
- Plasma cleaner: PDC-3XG (Harrick)
- Vitrobot: Vitrobot Mark IV System (Thermo Fisher Scientific)
- Filter paper: Whatman 597 (Whatman)



Preparation of α -Syn Pre-Formed Fibrils from α -Syn monomers

- 1 To produce pre-formed fibrils for structural analysis, adhere to the standardized protocol outlined here: [Protocol for Generation of Pre-formed Fibrils] (<https://www.protocols.io/view/protocol-for-generation-of-pre-formed-fibrils-from-rm7vz3ezxgx1/v1>).

Plunging of α -Syn fibrils

- 2 Dilute α -Syn fibril solution to  0.5 μ L using the Tris-HCl buffer. 
- 3 To separate large clumps of α -Syn aggregates, sonicate the fibril solution for  00:10:00 on medium settings, 0.5 s ON/OFF in a water bath sonicator. 10m
- 4 Glow discharge grids for  00:00:40 in a plasma cleaner. The carbon side should point upwards. 40s
- 5 Prepare the Vitrobot for plunging.
- 5.1 Fill up the water tank, cool down the plunging stage, fill up the plunging cup with liquid 2:1 ethane/propane, insert blotting paper.

Note

To safeguard the Styrofoam blotting pads from potential contamination by α -Syn aggregates, it is advisable to use Teflon disks as separators between the pads and the blotting paper.

- 5.2 Set the plunging settings: Blot-force 7, Blot-time 5, 10°C, 100% Humidity.
- 6 Apply  3 μ L fibril solution on the carbon side of the grids, then immediately plunge.  

**Note**

Make sure to rigorously pipette up and down to mix solution immediately prior to application on the grids.

- 7 Store the grids in grid boxes in liquid nitrogen until clipping. From now on, the grids should always be immersed in liquid nitrogen!

Cryo-TEM data collection

8

Note

Steps are described for a Thermo Fisher Titan Krios microscope equipped with a Selectris energy filter, a Falcon IVi detector, and running EPU software.

Clip the grids to prepare loading the Titan Krios TEM.

Note

- Exercise caution and speed during this step, as most ice contaminations occur at this point.

- 8.1 Confirm that the carbon side is oriented upward in the clipped grid.

- 9 Using a loading station, load the clipped grids into the autoloader magazine.

- 9.1 Double-check the proper placement of the grids

- 9.2 Insert the magazine into the nanocab.

- 10 Insert the nanocab into the autoloader of the Krios and load the magazine.



11 Examine the grids to identify squares displaying holes filled with α -Syn filaments.



11.1 Squares with thicker ice usually contain more α -Syn filaments that were more evenly distributed over the entire hole.

11.2 If the ice is too thin, then it may happen that α -Syn filaments bundle together at the edge of a hole, leaving the middle region free.

11.3 Avoid grids with large, crowded aggregates (visible as shadowy spots even in higher magnifications).

Note

It might be possible to process a dataset containing those areas, too, if there are at least some areas where filaments do not overlap too much with each other.

12 Set up the automatic data collection using EPU.



12.1 Exclude holes with ice contaminations, dense aggregates, fissures, or those that are too close to the edge of the square.

12.2 Pixel size of 0.92 to 0.72 Å/px (130,000x to 165,000x magnification).

12.3 Electron dose of 40 e-/Å²

12.4 -2.4 to -1.2 μ m defocus range.

12.5 10 eV energy slit.

13 Calibrate the microscope.





- 13.1 Autoeucentric by beam-tilt.
- 13.2 Autofocus (over carbon).
- 13.3 Autostigmatate (over carbon).
- 13.4 Autocomma (over carbon).
- 13.5 Calibrate image shifts.
- 13.6 Using Sherpa and using an unobstructed beam (go to a hole in the grid).
 - Zero-loss centering of the energy slit
 - Tune Isochromaticity
 - Tune Magnifications
 - Tune Distortions
- 14 Collect 5,000 to 10,000 images.



Preprocessing

- 15 Motion correction and conversion of EER files into MRC files.

Note

Use a gain reference of the microscope the data has been collected on. These are used as a dark reference. If a gain reference is unavailable, an artificial reference can be generated by averaging and subsequently normalizing each image within the dataset. Nevertheless, the option to proceed without gain correction is also viable. Ultimately, the purpose of gain correction is to mitigate “shot noise,” which is averaged out similar to other types of noise.

- 15.1 Use MotionCor2 for motion correction and EER to MRC conversion.



- For more information, please consult the MotionCor2 which can be accessed at the following link: **MotionCor2 User Manual**.
- EER fractionation: Each EER frame group should receive $\sim 1 \text{ e}^-/\text{\AA}^2$ total dose.
- Use standard settings for motion correction, however, use 5×5 patches for enhanced local motion correction.

16 Import the images into RELION.

16.1 In the RELION project folder create a folder called "input". Then create a symbolic link to the motion corrected images within this folder.

16.2

Note

Alternatively, directly copy the images here, but symbolic links typically function just as effectively, allowing the storage of images elsewhere, such as in a designated "RAW_DATA" folder.

16.3 Within RELION, create an Import job. Select "input/*.mrc" to designate raw input files (the wildcard "*" includes all .mrc links in the input folder). Ensure accurate entries for pixel size, voltage, spherical aberration, and amplitude contrast. Additionally, supply an MTF file for the detector used in data acquisition to enhance processing precision.

17

Note

As an alternative, the internal motion correction feature in RELION can be utilized. This also facilitates per-particle motion correction in subsequent stages, commonly referred to as "polishing." In brief, the EER files should be imported into RELION, followed by motion correction. Subsequently, the processing can be continued as outlined below.

18 Correct the CTF.

18.1 Use CTFFIND4.1 on dose-weighted images with standard settings (spherical aberration of 2.7, amplitude contrast of 0.07). Additional parameters involve a box size of 512, resolution limits of 30 Å (lower) and 4 Å (higher), and a defocus search range spanning from 3000 to 50,000 Å in 500 Å increments (100 Å steps for astigmatism).



Particle picking in crYOLO

19 Particle picking generally adheres to the crYOLO filament data picking tutorial, which can be accessed at the following link:

https://cryolo.readthedocs.io/en/stable/tutorials/tutorial_overview.html#tutorial-3-pick-filaments-using-a-model-trained-for-your-data

19.1 Manually pick filaments on 50-100 micrographs.

19.2 Use those particle coordinates as training data for the crYOLO network.

19.3 Use the trained network to predict particle / filament positions on all micrographs.

19.4 Using the crYOLO boxmanager, check if the network picked reasonable filaments, if not try to increase the training data and repeat.

19.5

Note

Particle picks on carbon edges or other equally undesirable locations can be filtered out during 2D classification.

20

Note

Alternatively, filaments could be picked entirely manually. Depending on the dataset, this process might take a considerable amount of time, but a better and more comprehensive particle set will be obtained, along with valuable insights into the data.

21 Importing particle coordinates into RELION exploiting RELIONs manual picking interface.

21.1 Start a manual picking job in RELION.

- Use the CTF-corrected images.



- Choose start-end coordinates of helices.

- 21.2 Pick filaments on a single image, save the coordinates, and then end the job. This will create the directory environment necessary for the import of crYOLO coordinates.
- 21.3 Go to the output folder of the Manual picking job and delete all .star files.
- 21.4 Copy all the .star files from the STAR_START_END output folder of crYOLO into the output folder of the Manual picking job.
- 21.5 Rename the .star files in the Manual picking output folder from *.star to *manualpick.star. RELION will now recognize the crYOLO coordinate files.
- 21.6 Continue the Manual picking job. The manual picking interface should show the filaments that were picked by crYOLO. Confirm whether the network was picked reasonably. If not, then train the network on a bigger training set. See Figure 2A for an exemplary picked micrograph.

2D classifications to get a good particle set - Binned initial 2D classification

- 22 Extract binned particles.
 - 22.1 Use imported crYOLO coordinates as particle set (from the fake Manual picking job) and CTF-corrected images.
 - 22.2 Extract with a large box size (756 px), then downsample 3 to 6 times to speed up calculations and signal quality.
 - 22.3 Make sure to switch on helical extraction. For amyloid helices, the rise is usually 4.75 Å, and following Lövestam & Scheres, extract every third unique asymmetric unit.
 - 22.4 Settings:
 - **Extract**
 1. Particle box size: 768
 2. Rescale particles: Yes
 3. Rescaled size: 256 (bin3)
 - **Helix**
 1. Extract helical segments: Yes
 2. Tube diameter: 200 (whatever is reasonable)
 3. Number of unique asymmetrical units: 3



4. Helical rise: 4.75

23 2D classification

23.1 Use the extracted binned particles.

23.2 Settings:

1. CTF

- Do CTF-correction: Yes
- Ignore CTFs until first peak: Yes

Note

Ignoring CTFs until the first peak acts as a high-pass filter, which tends to help align amyloid helices.

2. Optimization:

- No. classes: Usually so that I have 1.000 to 10.000 particles per class up to a maximum of 100 classes.
- Reg. param. T: 2
- Use EM algorithm: Yes
- Mask diameter: $0.95 * \text{pixel size} * \text{box size}$

3. Sampling

- Perform image alignment: Yes
- In-plane angular sampling: 6
- Offset search range: 5
- Offset search step: 1
- Allow coarser sampling: No

4. Helix

- Classify 2D helical segments: Yes
- Tube diameter: 200
- Do bimodal angular searches: Yes
- Angular search range – psi: 6
- Restrict helical offsets to rise: Yes
- Helical rise: 4.75

5. See Figure 2B for exemplary binned 2D classes.

24 Selection of good particles

- 24.1 At this stage, only classes containing good particles will be chosen (see Figure 2B, panels 2-4). That is classes whose class average distinctly resembles a helical tube of an amyloid protein. Ensure that homogeneous groups are selected, for example, refraining from mixing fibrils containing multiple protofilaments (Figure 2B-2 and 2B-4) with single protofilament fibrils (Figure 2B-3).

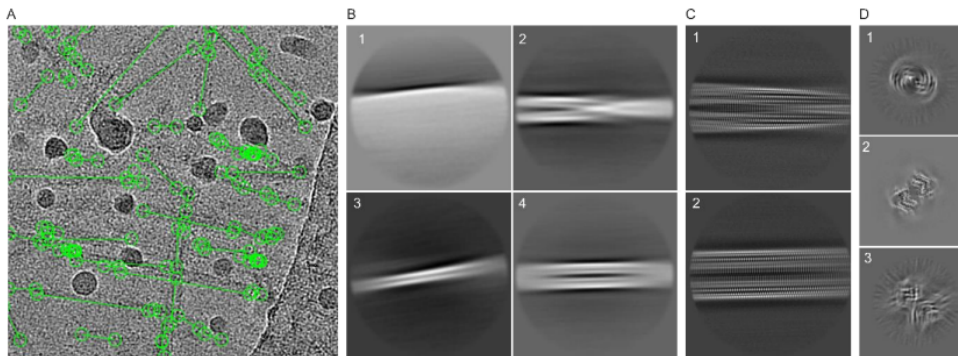


Figure 2: Exemplary pictures for various data processing steps. A) TEM micrograph showing α -Syn filaments that were picked by the crYOLO network (green lines). Circles show the start and end points of filaments. B) Binned class averages of 2D classifications. 1) Class average of carbon edges, which can be discarded. 2) and 4) Class average of α -Syn filaments consisting of two protofilaments, which can be grouped together. 3) Class average of single protofilament α -Syn filaments. C) Class averages of unbinned particles showing the amyloid typical beta-sheet separations: 1) shows a twisting filament, which can be further processed, while 2) shows a non-twisting class average, which may not be used for helical processing. D) 3D classification results with typical artifacts arising from a twist setting that is too high (1), hence the map is smeared in a circle, or too low (3), where density streaks in the solvent area can be observed. If the twist was close to the actual twist of the amyloid helix, the arising map shows clear peptide backbone structures (2).

2D classifications to get a good particle set - Unbinned 2D classification

- 25 Extract unbinned particles. Use a box size of 256 to 384 px.



26 Run another 2D classification.

26.1 Settings:

1. Optimization:

- Decrease the class number and mask size according to the box size used.
- Increase the number of iterations if the algorithm does not converge within 25 iterations.

2. Sampling:

- In-plane angular sampling: 1
- Offset search range: 10
- Offset search step: 1
- Allow coarser sampling: No

27 Selection of beta-sheet classes.

27.1 At this stage, select only classes that contain beta-sheet separated classes. Again, make sure not to mix different polymorphs together into the same selection as this will prevent obtaining a good 3D class later down the line. See Figure 2C for exemplary 2D classes showing beta-sheet separated helices.

3D classification - Find the helical parameters

28 If the crossover distance is visible from the micrographs or from binned 2D classes, use that to conveniently calculate the helical parameters. Otherwise, use a brute-force approach to find the correct helical parameters.

29 **Brute force option #1: Use `inimodel2d` to generate a range of initial models.**

This is the computationally more parsimonious brute force method. Use class averages that show clear beta-sheet separated filaments with a range of different possible crossover distances to generate initial models using RELION's `inimodel2d` program. If the crossover is correct and the averages cover most of the helix, then the resulting initial model may reveal a surprising amount of detail about the amyloid structure. Even in the absence of clear polypeptide backbone signals, arising artifacts give hints whether the crossover used (i.e., the underlying twist) is too large or too small which allows narrowing down the possible twist range. See Figure 2D for exemplary 3D classes showing the distinct artifacts arising from false twist settings.

30 **Brute force option #2: Use 3D classifications directly with range of twist**

An alternative approach is to opt for 3D classifications directly, systematically

testing various settings for the twist through brute force. Depending on the specified twist range and step size, this necessitates executing 10-20 3D classifications, which comes at a significant computational expense. Like the results from the inimodel2d program, results from 3D classification may hint at the correct twist depending on arising artifacts (see Figure 2D).

3D classification -Run the 3D classification

31 Use the beta-sheet separation showing classes as input particles.

32 Settings:

32.1 Reference

- Reg. map is on absolute greyscale: No
- Initial low-pass filter: 25
- Symmetry: C1

32.2 CTF

- Do CTF-correction: Yes
- Ignore CTFs until first peak: Yes

32.3 Optimization

- No. of classes: 3-4
- Reg. param. T: 15-30 (Note: This helps to align amyloid particles. However, keep in mind that the resolution estimates between the iterations are highly overestimated and that there is a risk of overfitting the data!)
- No. of iterations: 25-35 (depending on whether the algorithm can converge on a structure within the number of iterations provided).
- Mask: 0.95*box size*pixel size

32.4 Sampling

- Angular sampling interval: 1.8 degrees
- Offset search range: 5
- Offset search step: 1

32.5 Helix

- Do helical reconstruction: Yes
- Tube diameter (inner, outer): -1, 180
- Apply helical symmetry: Yes
- Number of unique asymmetrical units: 3



- Initial twist: Use different twists if you want to brute force, otherwise use a twist valuable calculated beforehand.
- Initial rise: 4.75 Å
- Central Z length: 30%

3D classification - Select particles belonging to high-resolution 3D class

- 33 Select classes that at least shows hints of the polypeptide backbone when looking at Z slices of the resulting model. An example of a good 3D class can be found in Figure 2D-2.

3D Refinement and postprocessing - Initial 3D autorefinement

34

Note

In initial 3D refinement runs, it might be beneficial to consider elevating the regularization threshold T (experimenting with values between 2 and 50). It is important to note that resolution estimates become unreliable in this scenario, and there is a potential risk of overfitting the data. Nevertheless, this adjustment could aid the algorithm in aligning amyloid particles.

- 35 Select particles and map from best class of previous 3D classification as input particles and initial model.

- 36 Settings:

36.1 Reference

- Absolute greyscale: Yes
- Initial low-pass: 20
- Symmetry: C1

36.2 CTF

- Do CTF-correction: Yes
- Ignore CTFs until first peak: Yes

36.3 Optimization

- Mask diameter: Pixel size * box size * 0.95

36.4 Auto-sampling



- Initial angular sampling: 1.8 degrees
- Initial offset range: 10
- Initial offset step: 1
- Local searches from auto-sampling: 1.8 degrees
- Use finger angular sampling faster: No

36.5 Helix

- Do helical reconstruction: Yes
- Tube diameter (inner, outer): -1, 180
- Apply helical symmetry: Yes
- No. unique asymm. Units: 3
- Initial twist & rise: same as 3D class., 4.75
- Central Z length: 20-30%
- Searches: No

3D Refinement and postprocessing - Create a mask

37 To generate an appropriate mask for postprocessing, fine-tune the initial binarization threshold.

37.1 Download the best class from the previous successful 3D classification.

37.2 Apply a low-pass filter to 15 Å (using `relion\_image\_handler -i 3D\_classification\_map.mrc--o lpf15A\_3D\_classification\_map.mrc--lowpass 15`)

37.3 Open the filtered map in Chimera. Inside Chimera, adjust the threshold settings until no "dust" particles are visible.

38

Note

It is advisable to generate multiple masks. For instance, consider creating "soft" masks by extending the initial map by a certain number of voxels (e.g., 5-7) and adding a soft edge of a specified voxel count (e.g., 5-7). On the other hand, "hard" masks can be created with minimal additional voxels and soft edge voxels (0-5). Given the variability in samples, experimenting with different maps is recommended, offering flexibility to explore diverse outcomes.

39 Settings

39.1 Mask

- Lowpass filter: 15
- Initial binarization threshold: Take from chimera
- Extend binary map: 0-7
- Soft-edge: 0-7

3D Refinement and postprocessing - Postprocessing

- 40 Apply postprocessing to enhance the quality of the map and achieve a more accurate resolution estimate, particularly if the regularization parameter T was adjusted during autorefinement.
- 41 Use one of the two half-maps obtained from the 3D autorefinement in conjunction with the generated mask for postprocessing. Make sure to input the MTF of the detector in the "Sharpen" tab.

3D Refinement and postprocessing - Iterative refinement runs

- 42 To further enhance the quality of the map, refinements and postprocessing can be conducted iteratively, typically 2-3 times. This process may include the utilization of symmetry (commonly observed in amyloids, such as C2 symmetry) or optimizing helical parameters through local searches in the "Heli" tab. It also makes sense in later refinements filter applied to increase the resolution of the initial model by decreasing the lowpass filter applied (e.g., to 10 or 5 Å).

Enhancing the quality of the map / Postprocessing

- 43 CTF refinement
- 43.1 To increase the quality of the map even further, refine the CTF for each particle using CTF refinements. Use the particles from the latest 3D refinement job and the postprocess.star file from the latest postprocessing job.
- 43.2 1st Refinement: Anisotropic Magnification, settings in the "fit" tab:
- Estimate (anisotropic) magnification: Yes
 - Perform CTF parameter fitting: Yes
 - Everything else: No
- 43.3 2nd Refinement: Defocus & Astigmatism, settings in the "fit" tab:
- Perform CTF parameter fitting: Yes
 - Fit Defocus: Per-particle
 - Fit Astigmatism: Per-micrograph
 - Everything else: No



- 43.4 3rd Refinement: Beamtilt, trefoil & higher order, settings in the “fit” tab:
 - Estimate beamtilt: Yes
 - Estimate trefoil: Yes
 - Estimate 4th order aberrations: Yes
- 44 3D refinement / 3D classifications
- 44.1 With the particles corrected for CTF, another 3D refinement is conducted, incorporating the search for helical parameters and exploration of various helical symmetries. Using the refined maps as an initial model, it is advisable to perform another 3D classification to selectively filter particles. Care should be taken not to excessively low-pass filter the initial model map during this process. Additionally, the particle stack can be filtered to exclude particles with very high defocus ($>20,000 \text{ \AA}$). This could speed up refinements/classifications without affecting the quality of the reconstruction too much.
- 45 Bayesian polishing
- 45.1 If RELIONs internal motion correction was used, Bayesian polishing can be utilized to increase the particle quality even further.

Protocol references

References

- Lövestam S, Scheres SHW. **High-throughput cryo-EM structure determination of amyloids**. Faraday Discuss. 2022;240(0):243-260. Published 2022 Nov 8. doi:10.1039/d2fd00034b
- MotionCor2 User Manual, hpc.nih.gov, <https://hpc.nih.gov/apps/RELION/MotionCor2-UserManual-05-03-2018.pdf>
- Polinski NK, Volpicelli-Daley LA, Sortwell CE, et al. **Best Practices for Generating and Using Alpha-Synuclein Pre-Formed Fibrils to Model Parkinson's Disease in Rodents**. J Parkinsons Dis. 2018;8(2):303-322. doi:10.3233/JPD-171248
- Protocol for Generation of Pre-Formed Fibrils From Alpha-Synuclein Monomer, [www.protocols.io](https://www.protocols.io/view/protocol-for-generation-of-pre-formedfibrils-from-rm7vz3ezxgx1/v1/protocols), <https://www.protocols.io/view/protocol-for-generation-of-pre-formedfibrils-from-rm7vz3ezxgx1/v1/protocols>, doi: 10.17504/protocols.io.bws6pehe
- Scheres SH. **RELION: implementation of a Bayesian approach to cryo-EM structure determination**. J Struct Biol. 2012;180(3):519-530. doi:10.1016/j.jsb.2012.09.006
- Scheres SHW. **Amyloid structure determination in RELION-3.1**. Acta Crystallogr D Struct Biol. 2020;76(Pt 2):94-101. doi:10.1107/S2059798319016577
- Tutorial 3: Pick filaments using a model trained for your data, cryolo.readthedocs.io, https://cryolo.readthedocs.io/en/stable/tutorials/tutorial_overview.html#tutorial-3-pickfilaments-using-a-model-trained-for-your-data
- Wagner T, Merino F, Stabrin M, et al. **SPHIRE-crYOLO is a fast and accurate fully automated particle picker for cryo-EM**. Commun Biol. 2019;2:218. Published 2019 Jun 19. doi:10.1038/s42003-019-0437-z
- Zheng SQ, Palovcak E, Armache JP, Verba KA, Cheng Y, Agard DA. **MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy**. Nat Methods. 2017;14(4):331-332. doi:10.1038/nmeth.4193