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Protocol status: In development
We are still developing and optimizing this protocol

Image processing of full-length monomeric LRRK2

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ABSTRACT

This protocol assumes that 2000 to 4000 movies of full-length LRRK2 embedded on vitreous ice are collected with an electron microscope equipped with a direct detector. It focuses primarily on the monomeric population of LRRK2 and has been leading to 3-4 Å resolution structures.

MATERIALS

CryoSPARC v4[1, 2]

Relion 4.03[3, 4]

Topaz 0.2.5[5]

UCSF Pyem 0.5[6]

ChimeraX[7]

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1 After movie alignment and CTF estimation (Patch Motion Correction and Patch CTF Estimation jobs in CryoSPARC), discard the micrographs with an estimated resolution worse than 4.5-5 Å.

1.1 We often perform these image pre-processing steps while the movies are being recorded, using CryoSPARC Live.

2 Initially pick your particles with Cryosparc's Blob picker.

2.1 Set particle diameter to 100-300 Å.

2.2 If using Cryosparc Live, calculate preliminary 2D classes to assess if you should proceed with your data collection.

2.3 You probably want to see at least 2 or 3 LRRK2-shaped 2D class averages after collecting 200-300 movies.

3 Extract your particles, binning them to a pixel size of approximately 4 Å and choosing a box size that encloses the whole LRRK2.

4 Perform rounds of 2D classification in CryoSPARC and select only the particles belonging to “good” 2D class averages.

4.1 The selection of “good” 2D classes depends on the user. Take into consideration the recognition of LRRK2 domains and the visualization of secondary structure elements. The number of particles of the class, the estimated resolution and CryoSPARC’s ECA parameter are helpful in sorting the classes to selection.

4.2 In the initial rounds, if in doubt of selecting a 2D class, it is OK to select it. Maybe you can rescue good particles?

5 With your best particles, train a particle-picking model using Topaz wrapper in Cryosparc.

5.1 Some parameters worth playing with are the Expected number of particles, Minibatch size, the Number of epochs and the Learning rate.

6 Extract the particles with a pixel size of around 4 Å.

6.1 Topaz Extract and Extract From Micrographs jobs.

7 Clean your particle dataset with several rounds of 2D classification in CryoSPARC.
Select the particles belonging to “good” 2D class averages.

7.1 We usually end up with more “good” particles after Topaz picking (by comparison with template-based picking like Blob picker).

8 Optional: remove bad particles by running an Ab-initio job in Cryosparc and discarding the particles belonging to the worst class, if it does not display LRRK2 features at all.

8.1 We introduced this step recently, with good results. It is a quick way of further cleaning the dataset.

8.2 We ask for a minimum of 3 Ab-initio classes, leaving all other parameters with default values.

9 Extract the best particles you have so far in CryoSPARC with your preferred box size.

9.1 We keep the pixel size ~ 4 Å at this stage.

10 Use the csparc2star.py command of UCSF pyem to convert the metadata of the extracted particles to the Relion STAR format.

10.1 UCSF pyem must be installed.

10.2 Example: csparc2star.py --swapxy YOUR_EXTRACTED_PARTICLES_FILE.cs YOUR_PASSTHROUGH_FILE.cs OUTPUT.star

10.3 The .cs files are inside the folder of the extraction job.

11 Create a Relion project directory.

11.1 Where you will run all your Relion jobs.

12 Run a 3D classification with alignment in Relion to further clean your dataset.

12.1 You need to edit the OUTPUT.star file to point to your extracted particles. sed command in Linux is a way of doing that.
Example: sed 's/J206/\/data\/leschzinerlab\/cryosparc\/P121\/J206/g' OUTPUT.star > OUTPUT1.star
(the above command is replacing J206 in OUTPUT.star for /data/leschzinerlab/cryosparc/P121/J206; in other words, you're pointing to the extracted particles with an "absolute" path that Relion can read from your Relion's project directory)

12.2 Since Relion only reads .mrcs files, you need to edit OUTPUT1.star to change .mrc to .mrcs.
Example: sed 's/particles\.mrc/particles\.mrcs/g' OUTPUT1.star > OUTPUT2.star

12.3 You also need to go to the Extraction job folder and change the .mrc extension to .mrcs. rename command in Linux is a way of doing that.
Example: rename .mrc .mrcs *.mrc

12.4 OUTPUT2.star will be the particle file inputted to the 3D classification job.

12.5 We usually use as initial reference the map of a full-length monomeric LRRK2, with the same pixel size and box of the particles. Both CryoSPARC (under Volume Tools) and Relion (relion_image_handler) can help you rescale, re-box and center your initial reference.

12.6 We recommend low-pass filtering the initial reference, either internally in Relion (you can use the GUI) or beforehand (with relion_image_handler, for instance); The default value of 60 Å should work.

12.7 In our experience, creating a soft mask around the reference usually increases the stability of the 3D classification (meaning that better volumes are obtained with fewer iterations).
To create a reference mask, we usually low-pass filter the reference (15 or 20 Å cut-off), display the map in ChimeraX and decide on a contour level threshold (no noise should be visible outside the volume or it should be erased). Then, we use Mask Creation in Relion to add a soft edge of 3 to 9 pixels (this is very important to avoid artefacts in Fourier space). Sometimes we extend the mask by 1 to 3 pixels.

12.8 Number of classes is a parameter you can play with, as the best results depend on each dataset (nature of the protein complex, homogeneity, how effective was 2D classification in “cleaning” the dataset, number of input particles, etc.).

12.9 We usually leave Regularization parameter T =4.

12.10 It can also be worth playing with the diameter of the circular mask applied to each particle. We tend to select a value that encloses all our protein, but depending on your goal the diameter may be decreased.

12.11 Sometimes limiting the alignment resolution to 6 to 12 Å yields better output volumes and 3D classification parameters (accuracy of rotations and translations and estimated resolution).

12.12 We usually run 25 iterations with default alignment parameters (angular sampling interval of 7.5 degrees, offset search range of 5 pixels, offset search step of 0.8). Then, we continue the job for further 25 iterations (or a minimum of 10 extra iterations) with finer angular sampling (angular sampling interval of 3.7 degrees, offset search range of 4 pixels, offset search step of 0.8 or 1). We regard this as a “polishing” step of your 3D classification. You should see the accuracy rotation and translation parameters improve, as well as relatively small changes in particle distribution. Most importantly, your output volumes should look “sharper” (compare iteration 25 with your final iteration, for instance), leading you to assume that the particles are being more precisely sorted between the number of classes you specified.

13 Display the output volumes in ChimeraX. Select the particles of the best volume(s) with Relion’s Select job.

14 Create a directory inside your Relion’s project directory with your aligned, CTF-estimated micrographs.

14.1 Usually, we create a symbolic link to each micrograph
Example: in the subdirectory that you just created, execute:
ln -sf
data/leschzinerlab/cryosparc/P121/S3/motioncorrected/*patch_aligned_doseweighted.mrc .
(Note that the final full stop above belongs to the command!)

15 Import micrographs to Relion with the Import job.

16 Extract the particles that you selected in step 13).

16.1 Using sed command, for instance, you need to edit the particle star file of your Selection job to change the path to the micrograph name.
Example: assuming your micrograph directory is dubbed “micros”, you need to change the “_rlnMicrographName” column to start with “micros/” followed of the name of each micrograph.

16.2 In the Particle extraction tab:
The micrograph star file is the one that you imported.
Input coordinates leave empty.
Your refined particles STAR file should be the one that you edited in the previous step. We usually extract the particles with a binning factor of 2 (around 1.9 Å per pixel).

17 Consensus 3D refinement.

17.1 Initial reference - the best class from 3D classification.
Although you can do it later, it is a good opportunity to change the handedness of your reference, if needed. You can do it in ChimeraX, Relion or Cryosparc.

17.2 We usually use a reference mask, prepared in a similar fashion to the described above.

17.3 Input particles are the extracted particles.

18 3D classification without alignment.

18.1

18.2 Input particles - the output data.star of the 3D auto-refine job.

18.3 Reference map - the output volume of the 3D refinement.

18.4 Since no alignments are calculated (the orientations are the ones found in the consensus 3D refinement), this job runs fast. We typically test the following parameters: number of classes, regularization parameter T, reference mask, low-pass filter applied to the reference, particle mask diameter.

18.5 Image alignment and GPU acceleration should be disabled.

18.6 Note that, depending on your goal, it might be useful to focus this classification on a particular region of LRRK2 by providing the appropriate reference mask.

19 After visualizing the output maps and analyzing key parameters, select the particles of the best class(es).

19.1 By key parameters we mean how the particles are distributed between classes, the accuracy of rotations and translations (smaller the better) and the estimated resolution. These parameters are helpful and often correlate with the quality of your maps.

20 Remove duplicate particles using the Selection job in Relion.

21 Import the particle coordinates using CryoSPARC's Import job.

21.1 The particle meta path should be the path to the particles without duplicates.

21.2 Enable the option “Ignore raw data”.

22 Extract the imported particles at the micrograph pixel size (without binning).

23 Non-uniform refinement in CryoSPARC.

23.1 Input particles - the extracted ones.

23.2 Initial reference - the best class from Relion, imported and re-scaled to the box size and pixel size of the extracted particles.

23.3 We typically test the parameters of this job.

23.4 We often obtain good results when enabling the option “Optimize per-group CTF params”.

24 3D classification without alignment in Relion using the orientations found in CryoSPARC’s Non-uniform refinement.

24.1 This step intends to discard particles, if any, that are not contributing to improving resolution.

24.2 You should use the command csparc2star.py of UCSF pyem to convert the particles of the last iteration of the Non-uniform refinement to Relion's star format, and use sed and rename commands.

24.3 Again, it is useful to play with 3D classification parameters and evaluate results. We tend to low-pass filter the reference less (if the resolution of the refinement was 4 Å, we test 6 to 15 Å, for instance, visualizing the effect of the low-pass filter on the initial reference).

25 Select the particles of the best classes, import the coordinates to CryoSPARC, extract the particles and run Non-uniform refinements.

26

Note

Note 1: consider retraining your Topaz model with the particles of the best map you have and repeat image processing.

Note 2: Depending on your goal, you might want to focus your 3D classifications and/or Refinements by applying a mask to your reference.
If focusing Refinements and 3D classifications are performed, consider trying also with signal subtraction at the particle level (of the signal outside the mask). We haven't so far seen
improvements with this strategy, though.

Note 3: Perform whole image processing inside Cryosparc too. We obtain good results very fast, and it is useful to decide if you want to apply the Relion 3D classifications described here to possibly improve your maps.

Note 4: Consider also 3DVA job in Cryosparc to simply record a movie or to sort out different states of LRRK2, as well as tools as cryoDRGN.