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Processing and symmetry expansion of LRRK2^{RCKW} on microtubules

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1

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Protocol for symmetry expansion of LRRK2^{RCKW} filaments bound to microtubules. This protocol covers everything from preprocessing to creating a new set of symmetry expanded particles that can be used by any of the popular cryo-EM refinement programs.

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Keep in mind that filament information contained within the Relion star files is important in preventing duplicate particles. CryoSPARC recently included helical processing, but I did not have time to verify if helices extracted using it will contain the proper helical information for Relion to work with.

Relion does good job of preventing duplication as long as Helical Reconstruction is on (even if not using helical symmetry). If FSC do not reach 0, there is a good chance that something went wrong and duplicate particles were created along the way. Once symmetry expanded, there is no easy way to check for particle duplication.

Requires the use of Relion 3.1 for the new particle subtraction job.

Software for mask manipulation. We used Python with mrcfile and numpy packages installed, but similar results can be achieved in Chimera or ChimeraX if creating the masks by hand.

None.

Preprocessing data

1 Align frames and do CTF fitting

Use your preferred software. The original publication used MotionCor 2 and CTFFIND4, but other programs should work just as long as CTF information can be imported into Relion. As a note, all of the data processed was collected on lacey carbon grids rather than C-Flats or UltrAuFolios.

Filament Picking

2 Manual picking to create references

We used Relion 3.0 originally (manual picking in version 3.1 should be identical) to manually pick filaments for about 20 micrographs. Using a helical rise of 30 Å, this resulted in about 1600 particles. This extraction used slightly modified extraction parameters from the rest of the data: 600 px box size, re-scaled to 250 px, resulting in 2.784 Å/px size. For helix settings, we used a tube size of 600 Å.

3 2D classification to create references

Using the extracted particles, we classified them into 10 classes, using 20 iterations, fast subsets off, and a 600 Å mask. For helix settings, I used a 600 Å tube diameter. Restrict helical offset to rise was not used. We selected 3 different looking classes to act as references for AutoPick.

4 AutoPick

References were lowpassed to 15 Å. In-plane sampling was set to 3 degrees. Autopicking threshold was set to 0.05. Minimum average noise was disabled and so was maximum standard deviation of noise. We used GPU acceleration with shrink factor of 1. Helix settings were: 500 Å Tube size and helical rise was set to 30 Å with 1 asymmetric unit. Maximum curvature was set to the default value of 0.1 and minimum length requirement was not imposed. These settings results in about 250 particles per micrograph, but were prone to picking carbon edges as well.

5 Extraction and particle clean-up

Initial extraction was done with a box size of 656 px, rescaled to 164 px (4x binning). This gave us a pixel size of 4.64 Å/px.

To clean up, we classified in Relion 3.1 with 100 classes, using 20 iterations (Fast subsets on), 450 Å mask, and initial angular sampling of 3 degrees.

Since this step is to mainly determine which particles have microtubules, the tube diameter was set to 300 Å and restrict helical offsets to rise was disabled.

Second round of the classification (50 classes, everything else the same) was performed on the good classes (anything that looked like it had a microtubule), removing further contaminants.

Selecting proper LRRK2^{RCKW} filaments

6 2D classification to select ordered LRRK2^{RCKW} filaments

50 classes, 25 iterations, fast subsets on, 650 Å mask, initial angular sampling of 3 degrees, 500 Å Tube diameter, restrict helical offsets to rise set to off.

Since this dataset contained MLI-2, only filaments showing extremely ordered LRRK2^{RCKW} were selected.

7 3D classification to determine protofilament size

This step is inspired by MiRP and used their provided references for the different protofilament sizes.

1 round of classification with alignment was done on the 6 references of protofilaments ranging from 11 to 16 PF size. The references were lowpassed to 15 Å, and a mask diameter of 450 Å was used. Sampling interval was set to 1.8 degrees. Helix settings were enabled without applying any helical symmetry. 325 Å tube diameter was used. Tilt and psi had a search range of 3 degrees.

The results split up the particles into different protofilament sizes. Only protofilament size of 11 was picked for this dataset.

Reference:

Alexander D. Cook, Szymon W. Manka, Su Wang, Carolyn A. Moores, Joseph Atherton, A microtubule RELION-based pipeline for cryo-EM image processing, Journal of Structural Biology, Volume 209, Issue 1, 2020, 107402, ISSN 1047-8477, <https://doi.org/10.1016/j.jsb.2019.10.004>

Code and references available at: <https://github.com/moores-lab/MiRP>

8 Re-extract particles at a lower binning

Initial re-extraction was done at 2x binning, but if computation power and storage is not an issue, you can re-extract at the original pixel size now, as the final symmetry expanded particles work best unbinned. Additionally, it is possible to continue using 4x binned images and unbin them at Step 15 as that is the last step before symmetry expansion.

Signal subtraction of the microtubule

9 Refine the microtubule section of the filament

Use the reference structure of a 11-PF microtubule lowpassed to 15 Å as a starting structure. We used a mask of 450 Å, and an initial sampling of 1.8 degrees.

Helix settings used a tube diameter of 300 Å, rot and psi search of 3 degrees, keep tilt-prior fixed set to true.

Helical symmetry applied was based on the usual 11-PF symmetry, in this case the starting point was 11 asymmetric units, -32.5 deg rotation and 11.3 Å rise. Twist search was confined to -33 and -32 degrees, but the rise was allowed to float between 10.5 and 12 Å. Both used 0.1 unit search.

If the final microtubule structure does not reach mid-resolution (about 6-8 Å or better), please do a 3D classification search with 3 classes, then use refine for each good class separately. However, the subtraction does not need to be perfect, as we will be reverting back to the original particles before the symmetry expansion.

10 Create a microtubule mask

Using the output of the refine, create a mask by choosing the lowest threshold that only shows the microtubule. We extended the map using 6 pixels, and added a 6 pixel soft-edge.

11 Subtraction

Due to changes to the subtraction module in Relion 3.1 (using half maps from the iteration, rather than the final map), we need to mimic Relion 3.0 behavior. This can be achieved in 2 ways:

1. Convert the star files to Relion 3.0 format and use the subtraction job there. Use the newly created mask and the final refine map.
2. Use the new External job type in Relion 3.1 to run subtract in legacy mode:

External executable: relion_project

Params:

i: final map from the 3D Refine

mask: newly created mask

ang: particle data from the 3D Refine job

o: name of folder as would be created by this job (ex. External/job024/subtracted)

angpix: -1 (or set to current pixel size, -1 will attempt to read the header)

subtract_exp

ctf

Old subtract only uses 1 thread, so leave mpi at 1.

Refining LRRK2^{RCKW} filaments

12 2D classification to choose the best LRRK2^{RCKW} filaments

40 classes (might need to adjust depending on the number of particles at this point, we had

47K for this particular dataset), 650 Å mask, 20 iterations, fast subsets set to off. 3 degree in-plane sampling. Tube diameter of 500 Å and no restriction on the helical offsets.
Only classes with an intact LRRK2^{RCKW} filament were picked.

13 3D classification of LRRK2^{RCKW} filaments

If no reference is available, run a Class3D job with a featureless cylinder with the expected diameter. Feel free to use a previously solved LRRK2^{RCKW} filament as a starting model if this is a related structure, as long as the protofilament size is the same. If running on a cylinder, only create 1 class. Otherwise 3 classes are better to use.

Settings to use:

Lowpass reference to 15 Å, 20 iterations, 650 Å mask, 1.8 degree angular sampling.

Helix settings:

Tube diameter 250-520 Å (to exclude potential left over microtubule signal)

3 degree tilt and psi search range, keep tilt-prior fixed true.

Helical symmetry settings:

33.3 degree rotation, 31 Å rise (or a previously refined value; starting values based on Villa's tomography paper)

Allow for symmetry search of +/- 2 degrees and 2 Å.

This might need to be iterated if some classes look less sharp than others. If starting with a cylinder, re-run this with the output but this time request 3 classes.

Reference:

Reika Watanabe, Robert Buschauer, Jan Böhring, Martina Audagnotto, Keren Lasker, Tsan-Wen Lu, Daniela Boassa, Susan Taylor, Elizabeth Villa,
The In Situ Structure of Parkinson's Disease-Linked LRRK2,
Cell,
Volume 182, Issue 6,
2020,
Pages 1508-1518.e16,
ISSN 0092-8674,
<https://doi.org/10.1016/j.cell.2020.08.004>

14 3D Refinement of the LRRK2^{RCKW} filament

Take the best LRRK2^{RCKW} classes and let them refine using Refine3D.

Use the same settings as the Class3D job above, updating the helical symmetry as needed.

15 Unsubtraction of the microtubule and full refinement

Use the subtract job to revert to the original particles. This should work even if the particles were created with the legacy Subtract job.

If planning on using unbinned data for the final symmetry expansion, make sure to re-extract the reverted particles with no binning at this stage, if you haven't done so before.

Use the reverted (and unbinned, if desired) particles for a final refinement of the filament:
Use the output of Step 14 as the reference, lowpassed to 15 Å and a 650 Å mask.
Make sure to set initial sampling to 1.8 degrees and that local searches is on at 1.8 degrees as well. This should cause Relion to start off with a local search rather than randomizing the poses, as we need keep the particles in roughly the same position as in the last step.
Use the full tube diameter of 520 Å (we are refining the microtubule as well in this step).
Angular search should still be 3 degrees for both tilt and psi.
Since the microtubule does not have the exact symmetry as the LRRK2^{RCKW} filament, we are not applying helical symmetry for this step.
If everything goes well, you will have a map with both LRRK2^{RCKW} and a low resolution microtubule visible.

Symmetry expansion

16 Create masks for LRRK2^{RCKW} dimers within the filament.

Since in our case there were 3 strands of LRRK2^{RCKW} present that did not follow C3 symmetry, we had to create masks for each LRRK2^{RCKW} dimer we wanted to extract. We chose to focus on the CorB-CorB dimer as the center of the mask and kept a significant portion of the microtubule within the mask as well. Parts of the neighboring LRRK2^{RCKW} were kept as well. Since each "particle" mrc is just a moving frame of 30 Å along the filament, make sure that the three masks you are creating are relatively planar to each other.
To facilitate creations of the masks, we created spherical "ones" masks for each dimer we wanted to extract and then multiplied it by the refined mask. Note: this behavior is new to Relion 3.1, as previously masks were used to determine what to subtract, but now they are used to determine what to keep, hence the "ones" map.
Each resulting mask was the processed by Relion by lowpassing to 15 Å, extending the map by 12 pixels, and adding a 12 pixel soft-edge. Helix option is not used for mask creation.

17 Symmetry expansion by subtraction

Using Relion 3.1's new Subtract job, we used the last optimizer from the previous refine job as input and created a Subtract job for each mask created in the last step. We also used centered the images on the mask and changed the output size to be smaller, 300 by 300 pixel particles if using unbinned particles, as this saves on computational processing power later on.

18 Realign the new particles

Since each mask corresponds to a different orientation of LRRK2^{RCKW} within the filament, they need to be realigned. For best results, use one of maps that was used to create the subtraction masks. It will need to be same size as the new particles. We used python scripts to extract a subsection of the map centered on the mask. Alignment can be done in any processing software. We transitioned almost exclusively to CryoSPARC at this stage, and from this step processing will vary depending on what one wants to accomplish.