Jun 21, 2024 Version 2

Tomogram reconstruction and sub-tomogram averaging to obtain full-length, auto-inhibited LRRK2 filaments on microtubules V.2

DOI
dx.doi.org/10.17504/protocols.io.n92ld89zxv5b/v2

Siyu Chen[1], Josh Hutchings[1], Digvijay Singh[1]
[1]UC San Diego, HHMI

ASAP Collaborative Rese…

Siyu Chen
UC San Diego, HHMI

DOI: dx.doi.org/10.17504/protocols.io.n92ld89zxv5b/v2

Protocol Citation: Siyu Chen, Josh Hutchings, Digvijay Singh 2024. Tomogram reconstruction and sub-tomogram averaging to obtain full-length, auto-inhibited LRRK2 filaments on microtubules. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n92ld89zxv5b/v2Version created by **Siyu Chen**

Protocol status: Working
We use this protocol and it's working

Created: April 22, 2024

Last Modified: June 21, 2024

Protocol Integer ID: 102234

Keywords: ASAP LRRK2

Funders Acknowledgement:
asap
Grant ID: ASAP-000519
National Science Foundation
Grant ID: DBI 1920374

Abstract

This protocol describes all the data analysis steps after obtaining the LRRK2 on microtubule dataset.

Tilt series alignment and tomogram reconstruction

1 Use Warp for the pre-processing of tomograms before subtomogram analysis.

Software	
Warp	NAME
Windows 10	OS
Dimitry Tegunov	DEVELOPER

Perform motion correction, CTF estimation and gain correction in the '.tif' page. Set the pixel size, dimensions, binning (usually un-bin), CTF searching range, number of frames in the corresponding places.
Averaged tilts after motion correction are saved in the 'average' folder under the data folder.

2 After motion correction is done, go to '.tomostar' page and click "Import Tilt Series from IMOD" to generate tilt stacks suitable for tilt series alignment in eTomo
Select the data folder and metadata folder (where .mdoc files are saved for all tilt series) then click "Create stacks for IMOD".
Warp will now create an 'imod' sub-directory within the project directory and save the .st stack file and its corresponding .rawtlt file

3 Align all tilt series in eTomo in IMOD using patch tracking.

Software	
IMOD	NAME
Linux	OS
University of Colorado Boulder	DEVELOPER

Using command line run 'etomo' and click 'Batch Tomograms' to process all tilt series.

4 In the first page (Batch Set up), choose how the stacks are saved (in 'imod' folder or already in subfolders) accordingly and select the cryoSample.doc template in 'System template'.. Then move to 'stacks' folder to import all stacks ready for alignment

4.1 If there are bad tilts that were identified previously, they can be removed now by excluding the tilt number in the 'stacks' page.

5 The 'Dataset Values' tab contains all of the parameters needed for patch tracking. In either 'Basic' of 'Advanced' value pages, make sure to find and set the following:
Bin 2 or 4 for coarse alignment;
Use patch tracking with patch size of 170,170 or 340,340;
Tomogram thickness 1500 - 2500 pixels
Pixel size and Tilt axis rotation angle obtained from the data collection parameters
Microscope voltage and Cs (also read headers from the image file -- serve the same purpose)
Patch overlap 0.33,0.33 (percentage)
Make sure magnification, tilt angle, rotation and beam tile are NOT aligned (choose 'fix' for all)
Use Robust fitting
One can change other parameters based on needs or leave them as blank or default

6 When everything is set up, run the batch job in the 'run' page.
It is optional to reconstruct a tomogram at this stage. Choose 'stop after Positioning' to skip it.

7 When finished, check the log file in each subfolder to obtain the Residual error mean (in nm) value. It is used to assess the alignment quality for different tilt series. Tomograms with <2 nm Residual error mean are eligible for future analysis.
If the value is too big, something is off during the alignment. Use etomo to open the '.edf' file in the corresponding folder to check the patch alignment contours in the 'Fiducial Model Generation (Patch-tracking)' page and use ctrl+D to delete bad tracks in the pop-up IMOD model window.

Note

Alternatively, a boundary model can be generated in this page as an IMOD close countour model. Such a model should exclude all carbon substrate areas and empty areas in the tomogram because microtubule sample often contains them. Carbon substrate areas and empty areas severely mess up the patch tracking accuracy.

8 When alignments are all examined and optimized, use the eTomo alignment data (the .xf file in each subfolder) in Warp for tomogram reconstruction.
In the same "Import Tilt Series from IMOD" page, claim frame folder, metadata folder, alignment file folder (the 'imod' folder containing all .xf files) and put in the estimated dose. The click 'import'.

9 Estimate 3D CTF of all tomograms and reconstruct it using weighted back-projection at 10 Å/pxl. Tomogram visualization and microtubule tracing will be performed on de-convoluted tomograms generated from Warp with default settings.

Microtubule tracing, LRRK2 sub-tomogram picking and ab-initio model building in Dynamo

10 IMOD was used to manually go through all reconstructed tomograms and trace the backbone of microtubules. For each intact microtubule, make an open trajectory model by pressing the middle mouse button along the microtubule axis and save the corresponding '.mod' file that saves the x-y-z coordinates of every points.
Only microtubules with LRRK2 decoration and without extensive overlapping were selected for further analysis.

11 Subject the coordinates of selected microtubules to Dynamo in Matlab (https://www.dynamo-em.org/w/index.php?title=Main_Page) and use the dmodels.filamentWithTorsion() function to re-sample equidistantly for sub-tomogram extraction from the reconstructed tomogram.

Software

Dynamo	NAME
Linux	OS
CU Boulder	DEVELOPER

Meanwhile, the azimuth angle of the picked sub-tomograms which rotates around the microtubule was randomized using the function dtrandomize_azimuth() to minimize the effect of the missing wedge. The result table was used to crop sub-tomograms using the function dtcrop().
After the extraction, sub-tomograms were saved in a dedicated folder and their average without alignment can be calculated using daverage(). The box size was 48 for this step.

12 Align the picked sub-tomograms with the featureless tubular reference generated from the last step using the function dcp.new() (create the project) and dvput() (set parameters).
Local parameters limit the first two Euler angles (cr_r, cone range, 20) and movements (lim_r,shift limits,[10,10,2]) along the microtubule were applied for searching.

13 Removed duplicate sub-tomograms based on distance using the function dpktbl.exclusionPerVolume(), and smooth the coordinates again to be equidistantly distributed by averaging the coordinates of the adjacent sub-tomograms in the table.
Each data point here would be used and the anchor point to geometrically search LRRK2 sub-tomograms around microtubules.

14 Pick sub-tomograms in rings orthogonal to the path, pointing outwards using the function dmodels.filamentRings().
LRRK2 sub-tomograms were over-picked, with a radius of
23 nm to the center, particle separation of 7 nm between ring layers, and 18 subunits per ring. With this set of parameters, both LRRK2 and about half of the microtubule filaments are included with a particle box size of 36 nm.

15 Manually scan through the picked microtubules to check the picking result and choose several microtubules with relatively full LRRK2 decorations to obtain the initial model.
Sub-tomograms from such microtubule can be aligned using the same set of parameters for 10 iterations (ite_r, 10) without any initial reference.
The selected initial, low-resolution model will be used for all sub-tomogram analysis in later steps.

16 Using the same function, all LRRK2 sub-tomograms were then aligned to the reference for only one iteration in Dynamo, and both duplicated sub-tomograms as well as sub-tomograms having lower than 0.32 cross-correlation value against the reference was removed.

17 The averaged density from each microtubule was manually examined at the same time using IMOD to check the skewing direction of tubulin filaments from z-direction.
The orientation of traced microtubules was random, so sub-tomograms with opposite skewing directions should be flipped in Dynamo to align microtubules better. Add 180° to the 'narot' Euler angle in the 9th row of the table to flip the microtubule orientation.

18 Center the picked tomograms to either the WD40:WD40 interface or COR:COR interface as needed, by manually re-sampling the averaged map in Chimerax and use dynamo_align() function to move the sub-tomograms in the stack accordingly.

Software	
Chimerax	NAME
Mac	OS
UCSF	DEVELOPER

19 The aligned LRRK2 sub-tomograms were then subjected to 4 iterations of rough, local refinement in Dynamo (cone range 30; in-plane range 30; shift limit [4,4,4]).
The resulting sun-tomogram coordinates can be checked using Artiax in Chimerax, which should look regular but scarcely distributed around the microtubule.

Sub-tomogram analysis and three-dimensional model reconstruction of LRRK2

20 Use Warp to extract sub-tomograms using the "Reconstruct Sub-tomograms" function. The sub-tomograms were first binned by 2 (4.322Å/pxl) with a box size of 96

21 Use Relion3 to refine the sub-tomograms were refined with C1 symmetry. First without a mask, then the middle row of LRRK2 oligomer was masked to perform focused refinement.

Note

This step was skipped in the processing pipeline of two datasets. Dynamo stacks were directly used to extract bin-2 sub-tomograms in Relion4. After that, sub-tomograms were refined with a
full mask covering microtubules and the middle row of LRRK2 oligomers, then subjected to the same pipeline of Tomo-frame alignment, CTF-refinement, focused refinement, and 3D classification.
All of these variant pipelines resulted in comparable sub-tomogram maps of LRRK2 in the end. However, one should consider trying different combination of strategies for each individual projects.

Software

Relion	NAME
Linux	OS
MRC	DEVELOPER

22 To further improve resolution, Relion4 was used to extract sub-tomograms from the refined star file, then Tomo-frame alignment and CTF refinement was applied to optimize the tilt alignment for each tomogram.
The map resolution was improved to Nyquist (8.644Å) for all datasets after such refinement steps.

Note

One of our dataset shows stripe-like artifacts after initial refinement in Relion3, possibly from missing wedges. To overcome that, initial steps of pseudo-tomogram reconstruction in relion4 was performed with down-weighting particle signals in a 10° cone in Fourier space along the Z axis. The particle stacks were resumed to normal after CTF-refinement and the artifacts were not apparent anymore.

23 Next, focused refinement was applied to the middle 2 copies of LRRK2 followed by a 10-class 3D classification without any alignment.
To avoid over-fitting, the regularisation parameter T was set to 1 instead of default 4 and the resolution E-step was set to 20Å to disable alignment of high-frequency signals.

go to step #27 for the analysis focusing on microtubules.

Class-averages containing at least one good-looking LRRK2 were all chosen for further alignment were subjected to a C2 refinement.

go to step #40 This stack focuses on LRRK2 WD40:WD40 dimer sub-tomograms. It was used to perform the Geometric analysis of LRRK2 on microtubules

24 Next, one copy of LRRK2 was masked for further refinement, and the stack was symmetry expanded by 2-fold so that all LRRK2 copies can be included for alignment.
With this dataset, perform a 3D classification with the same set of parameters and pick good classes by carefully examining the class-averages.

25 Unbin he refined stack to 2.161 Å/pxl and perform another auto-refinement to improve resolution.

26 One can also try another round of Tomo-frame alignment with motions of each sub-tomograms estimated, then perform another auto-refinement to see if there is any improvement in resolution. Such procedure improved the alignment of one dataset in all our attempts and never decrease the resolution.

go to step #33 The same dataset was used to refine the novel WD40-ARM/ANK interaction surface.

Sub-tomogram analysis and three-dimensional model reconstruction of microtubules

27 From step 14, a C1 refinement focusing on only one copy of LRRK2 was performed to obtain microtubule sub-tomograms that have the same orientation.
With this dataset, perform a 3D classification with the same set of parameters and pick good classes by carefully examining the class-averages, similar as in step 15

28 Good sub-tomograms were subjected back to Dynamo and geometrically re-centered to focus on the microtubule it binds to. The z-direction of each particle was rotated as well, from being perpendicular to the microtubule to be parallel to the microtubule. The function dynamo_align() and dynamo_table_rigid() were used to rotate the orientation of sub-tomograms.
dynamo_subboxing_table() was used next to re-center each sub-tomograms to be centered on the microtubule.

29 Next, microtubule sub-tomograms were extracted in 10 Å/pxl and box size of 72 nm in Dynamo and aligned against a 13-pf microtubule initial model for 1 iteration.

30 The aligned stack was then subjected a 1-iteration multi-reference alignment in Dynamo using 11-16 protofilament microtubules as reference models. References were set as input using dynamo_write_multireference() and supplied to the alignment job similarly using dvpr()
Only sub-tomograms classified to the 13-pf class were used for the next steps.

31 The select stack was used in Relion4 again to extract bin-2 microtubule sub-tomograms with box size of 176 pixels. Using a feature-less tube as initial reference, perform Relion4 helical refinement (with tube
diameter 300Å, twist -27.7°, rise 9.4Å, central z-length 30%)

32 Un-binned sub-tomograms were then further refined with the same set of parameters, resulting in a 5.9Å microtubule map.

Sub-tomogram analysis and focused three-dimensional model reconstruction of LRRK2 WD40:ARM/ANK interface

33 A partial soft mask focusing on the ARM/ANK domain and the WD40 domain it interacts with (from the other copy of LRRK2) was applied to the LRRK2 map, and the sub-tomograms were re-centered, binned by 2, and re-extracted in Relion4 to perform focused refinement after getting the highest resolution map.

34 Next, a 3-class 3D classification was performed, resulting in one class showing extend density corresponding to the N-terminal region of LRRK2.
Such region remained flexible and was not resolved in the LRRK2 map.

35 Focused refinement with the un-binned stack gives improved resolution in the final map.

Model building

36 All published models of LRRK2 were used as references to build the LRRK2 model on microtubules. The main reference was PDB: 7LHW

37 First, since the overall structure of full-length LRRK2 fits well into the density map, ChimeraX was used to rigidly fit each domain of the in-solution LRRK2 model into the map.

38 Next, use ISOLDE in Chimerax to examine each connecting loop between domains and resolve the clashing and obvious wrong fitting with torsion and distances restrained.

Software	
ISOLDE	NAME
Mac	OS
Altos Labs	DEVELOPER

39 To model in the focused map showing WD40:ARM/ANK interface, the N-terminal region of LRRK2 was predicted using alphafold2 and fit into the map. ISOLDE was used again with restrains to resolve clashes at the conjunction between our LRRK2 model and the alphafold2 model, as well as fitting in a helix that was obviously out from the density.

Geometric analysis of LRRK2 sub-tomograms on microtubules

40 For the stepwise polymerization dataset, good LRRK2 sub-tomograms going into final map reconstruction were used, but the coordinates of each particle were extracted from the previous C2 refinement job
in the Relion pipeline because they correspond to a WD40-WD40 dimer of LRRK2.
From step 14, select sub-tomograms that went into the final reconstruction in step 17 and extracted in Dynamo as 10Å/pxl sub-tomograms.

41 Perform the same sub-boxing and refinement steps as step 19, then aligned for 1 iteration against a tubular reference.

42 Next, the distances and orientations of all LRRK2 particle pairs were calculated and plotted using their x-y-z coordinates. The LRRK2 sub-tomogram pairs that are roughly perpendicular to the microtubules were picked for further analysis.

43 Plot distances between all selected pairs first plotted to make sure LRRK2 was not over- or under-picked.

44 Next, the selected LRRK2 sub-tomogram pairs containing overlapping sub-tomograms can be grouped into chains. Each chain represents a short oligomer of LRRK2 with various copy numbers.
The number of LRRK2 dimer chains contain certain number of LRRK2 dimers were counted and plotted.

45 Finally, for each selected pair, the helical angle (θ angle) between the LRRK2 pair and the z-direction of
its corresponding microtubule particle was calculated in Dynamo and plotted.
The helical angle between to adjacent LRRK2 sub-tomograms can be calculated as the angle between two vectors: one connects the x-y-z coordinates of two LRRK2 sub-tomograms, the other is the z-vector of the corresponding microtubule sub-tomogram.