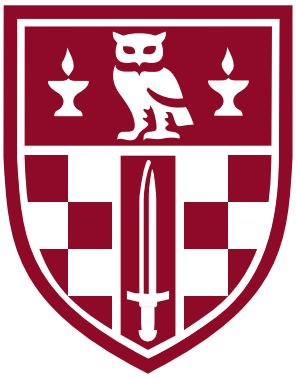
Manual



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Contents

1 Introduction

This manual gives instruction for using MiRP with RELION v3.1 to align asymmetric microtubules. It describes the use of MiRP within the RELION v3.1 GUI, but command line use is also possible. Users must at least have completed the RELION v3.1 tutorial before using MiRP, and at least have read the original MiRP publication (DOI: 10.1016/j.jsb.2019.10.004), as some working knowledge of both is required. The instructions here describe RELION settings relevant to MiRP only.

1.1 Requirements

MiRP uses Python3, with dependency on the numpy, scipy, and matplotlib modules. The easiest way to setup from scratch, is to install Anaconda. Some functionality requires EMAN2 (version 2.13 was used here), which is dependent on Python2. A PDF viewer is required to visualise MiRP diagnostics.

1.2 Overview

This manual is divided into four sections:

1. Protofilament number sorting.
2. Initial seam alignment.
3. Seam checking.
4. Tips/next steps.
5. Installation.
6. Citing MiRP.

2 Protofilament number sorting

2.1 Preprocessing

Only manually picked datasets have been used with MiRP so far. This is because auto-picking software tends to fragment tubes, ignore tube cross-overs, and pick random tube fragments. It is possible that some modern particle pickers such as crYOLO may be useable, since crYOLO applies post-hoc corrections to achieve smooth picking. Automated picking is however, untested as yet. We use manual picking in RELION or EMAN2, and try to be selective, as outlined in the MiRP publication.

After manual picking, perform particle extraction:

- Particle box size: 600 Å (7.4 αβ-tubulin dimers), although smaller box sizes may be necessary to avoid memory constraints when using smaller pixel sizes.
- Rescale particles? Yes. We perform initial alignments with binned 4 data.
- Extract helical segments? Yes.
- Tube diameter: 390 Å (depending on size/presence of decorating protein).
- Use bimodal angular priors? Yes.
- Coordinates are start-end only? Yes.
- Cut helical tubes into segments? Yes.
- Number of unique asymmetrical units: 1.
- Helical rise: 82.

2.2 Protofilament number classification

We use supervised classification to assign protofilament number to microtubules, based on the known helical architectures of 11-16 protofilament microtubules. Pre-generated references for these microtubule architec-

tures are supplied with MiRP in the data folder. We have found the presence/absence of decorating proteins in these references does not particularly influence the results. The references must have the same box and pixel size as your particles.

Use the 3D classification job type:

- I/O:
  - Reference map: pfrefs.star
- Reference:
  - Ref. map is on absolute greyscale? No.
  - Initial low-pass filter: 15.
  - Symmetry: C1.
- Optimisation
  - Number of classes: 6.
  - Number of iterations: 1.
  - Mask diameter: 90 % of box size.
- Sampling:
  - Angular sampling interval: 1.8.
  - Offset search range: approximately 84 Å
  - Offset search step: approximately 5 Å
- Helix:
  - Do helical reconstruction? Yes.
  - Tube diameter - inner, outer: 110, 390 (depending on presence/size of decorating protein.)
  - Angular search range - rot, tilt, psi: -1, 15, 10.
  - Range factor for local averaging: -1.
  - Keep tilt-prior fixed: Yes.

  - Apply helical symmetry? No.

2.3 Protofilament number correction

Now we can use MiRP to assess the number of particles in each microtubule that have been assigned to a particular protofilament number (class number), and calculate the most likely protofilament architecture for that microtubule.

Use the External job type:

- Input
  - External executable: mirp_pf_sorting
  - Input particles: input the run_it001_data.star file from your protofilament number classification job.
- Params
  - Param1 label, value: reset_eulerxy
  - Param2 label, value: conf, 75

The reset_eulerxy flag sets the euler angles and xy shifts to zero or to priors, which is helpful for the next step. The optional conf flag specifies a confidence threshold (1-100 %), where microtubules that have a confidence in protofilament number assignment below this threshold will be removed. When the job is executed, a progress bar appears in the RELION standard out window, and when the job has finished, some information about the run appears. There are now four checks to carry-out:

- Different microtubule preparations result in different populations of protofilament numbers. Check the table in the RELION standard out window, and see if the protofilament number distributions are what you expect, for example if you have taxol stabilised microtubules, but see a large amount of 11 protofilament number microtubules, then something is probably wrong. The most common issue is that the references used for 3D classification are not the

Protofilament number	Taxol (%)	GMPCPP (%)
11	0.3	0.5
12	4.7	0.7
13	44	8.3
14	41	83.5
15	6	2
16	4	4

Table 1: Example distribution of different protofilament number microtubules in taxol and GMPCPP datasets.

correct pixel size/box size, or have been rescaled incorrectly. Table 1 shows what you should expect from taxol stabilised or GMPCPP microtubules:

- Take a look at the file protofilament_corrected.pdf, which will be in the folder: External/job_id/protofilament_corrected.pdf. This file has a page for each microtubule in your dataset, where the graph on the left shows the profilament class number (1-6 corresponding to 11-16 protofilament microtubules) over microtubule particle number, plotted for the RELION protofilament classification job we previously carried out. This plot should show various protofilament class assignments for microtubule particles, however it should be obvious that there is a predominant class number (see figure 1 for an example). The plot on the right shows the MiRP corrected class assignments for that microtubule. Take a look through the different microtubules. Is there a predominant class type for each microtubule, or are the class assignments quite varied? The latter indicates that protofilament classification has not worked well. Where class assignments are too varied, and MiRP cannot decide what the helical architecture is, that microtubule will be discarded, and you will not see a graph on the right.

- Check the total predicted changes in protofilament number in the RELION standard out window. MiRP tries to detect where microtubules change protofilament number, for example in figure2, there is an obvious switch from 15 protofilament class assignments to 14.

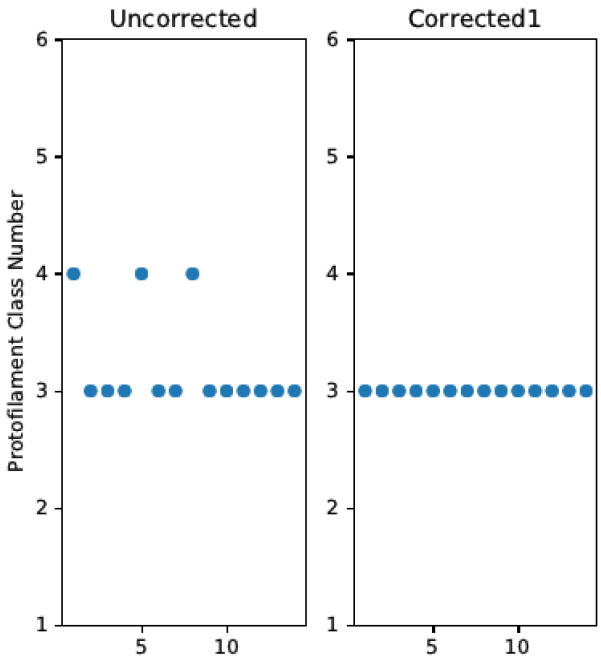

Figure 1: Protofilament class distribution for an example microtubule.

Accordingly, this microtubule has split into two new microtubules, as shown by the two plots on the right. This function is informative about data quality - MiRP will also split microtubules when it encounters a region of a microtubule that has variable class assignment - which could be caused by poor alignment or damage to this region of the microtubule. It is also quite common to observe so-called protofilament number changes at the ends of microtubules - which is most likely parts of that microtubule which are suboptimal, but have been accidentally included during picking. This is quite common in micrographs with many microtubules that are overlapping. The result is that the value for total predicted changes in protofilament number, should not be taken seriously, but a high value (more than 2-5 % of total microtubule number) indicates that the microtubule quality may not be good.

- Finally, take a look at the confidence.pdf file (again in the folder: External/job_id). MiRP calculates the confidence of each protofilament number assignment for each microtubule (defined as: num-

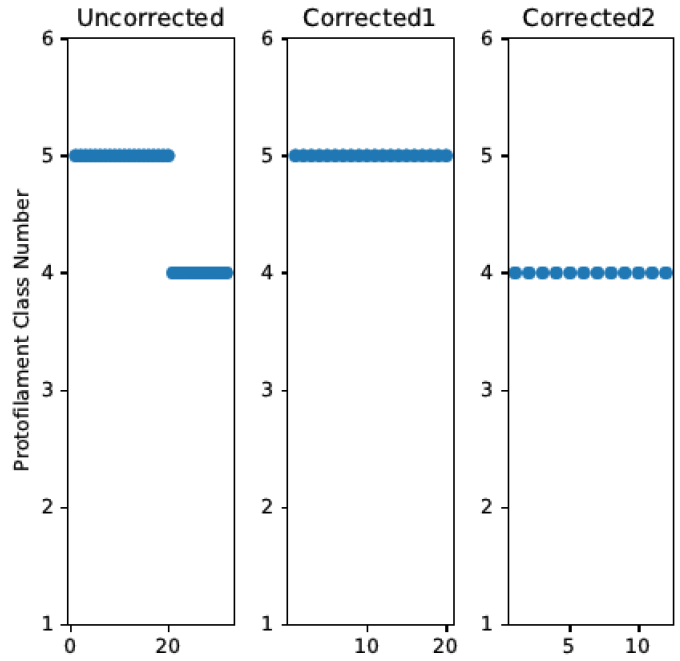

Figure 2: Protofilament class distribution for an example microtubule with a possible protofilament number switch. The microtubule is split into two separate microtubules, as shown by the two graphs on the right.

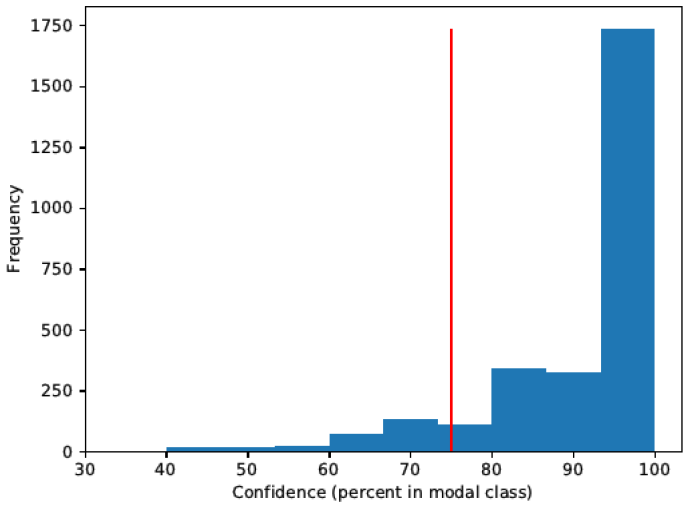

Figure 3: Confidence of protofilament number assignment for an example dataset.

ber of particles in modal class ÷ total number of particles × 100), and plots these values as a histogram in this file. Therefore, this graph shows you how successful protofilament number assignment has been for your data. The more of your data with 100 % confi-

dence the better, for example in figure3, nearly all the microtubules have been assigned protofilament numbers with 80 % confidence or higher. Protofilament assignment is usually very accurate, so you should expect to obtain a confidence distribution similar to this plot, with standard samples. The red vertical line indicates the confidence threshold cutoff, and microtubules with a confidence below this value will be discarded. After seeing this plot, you may wish to adjust your confidence threshold.

3 Initial seam alignment

Unless you have helical microtubules (15 and 16 protofilament number microtubules), your microtubules will suffer inaccuracies in alignment because of the asymmetric break known as the seam. The seam is difficult for software to detect, however your alignments will be improved if using a microtubule binding protein that binds a tubulin dimer. The following steps aim to achieve the best initial seam alignment.

3.1 Segment average generation

To improve the signal to noise ratio of individual particles, we average neighbouring particles in a microtubule over a sliding 7 particle window, to create segment averages. This is done using EMAN2, whose libraries clash with RELION (the most common setup is to have a module system so that these programs can be loaded individually). For this reason, we load EMAN2 and run this step from the command line, for example:

```
generate_segment_averages -i ./External/mirp_pf_sorting/14pf_data.star -o ./Extract/segmentAverages
```

A new folder will be created as specified with the -o flag, with a directory containing the segment averages, and a particles.star file for input into RELION.

3.2 Rot angle assignment

The particles from one microtubule should all share a very similar Rot angle, and for each microtubule, there is only one Rot angle that will correctly align the seam in the experimental images, to that of the reference. However, for a 14 protofilament microtubule there are 14 local minima in the Euler sphere which produce very similar cross-correlation values. The result is that in practice, RELION will determine a wide distribution of Rot angles for a given microtubule. Our first job is to assign a Rot angle for each microtubule.

You will want a 3D reference corresponding to your sample at this point. For example in the data/initial_seam_references folder, there are various protofilament number microtubules with kinesin, or CAMSAP, which bind to quite different locations on the microtubule. It is important that you do not directly use the map from protofilament classification as a reference, since this will be a poor reference for the seam. **It is essential that your reference has the same polarity as the references in data/initial_seam_references, or the later seam checking step will not work.**

To perform the first alignment (global search), use the 3D classification job type:

- I/O:
  - Input: Extract/job_id/segment_averages.star
  - Reference map: e.g. 14pf_kinesin-3HQD_5-56A.mrc
  - Reference mask: Create a binary soft edge mask from your reference that is fairly loose. Central Z length = 90 %
- Reference:
  - Ref. map is on absolute greyscale? No.
  - Initial low-pass filter: 15.
  - Symmetry: C1.
- Optimisation
  - Number of classes: 1.
  - Number of iterations: 1.
  - Mask diameter: 90 % of box size.
- Sampling:
  - Angular sampling interval: 1.8.
  - Offset search range: approximately 84 Å

  - Offset search step: approximately 5 Å

- Helix:
  - Do helical reconstruction? Yes.
  - Tube diameter - inner, outer: 110, 390 (depending on presence/size of decorating protein.)
  - Angular search range - rot, tilt, psi: -1, 15, 10.
  - Range factor for local averaging: -1.
  - Keep tilt-prior fixed: Yes.
  - Apply helical symmetry? No.

Now perform Rot angle assignment using MiRP using the External job type:

- Input
  - External executable: mirp_initial_seam
  - Input particles: input the run_it001_data.star file from your global search job.
- Params
  - Param1 label, value: reset_xy
  - Param1 label, value: rot

A progress bar will appear in the RELION standard out, followed by some information once the job has completed. There are now some checks you can carry out:

- Open rotation_corrected.pdf. Every page corresponds to one microtubule. The left hand plot shows Rot angles plotted over particle number, and coloured lines that show clusters of Rot angles that MiRP has found, with the modal cluster being a bold line. The plot on the right shows the corrected Rot angles for the microtubule. An example of this can be seen in figure4. You can see that the

Rot angles are quite widely distributed, but that there is an obvious skew towards angles between -20-150°. The clusters are well defined, showing lines with shallow slopes, corresponding to the supertwist of this 14 protofilament microtubule. The top cluster is the line in bold, and has the most particles in it. The plot on the right shows the corrected Rot angles and you can see a nice line with a shallow slope. Rot angle assignment has not worked well if the Rot angles are very distributed, and there is no clear skew towards a certain range of values. Futhermore, if the clusters show lines with widely varying slopes, and there is no clear predominant cluster, then this indicates poor Rot angle assignment.

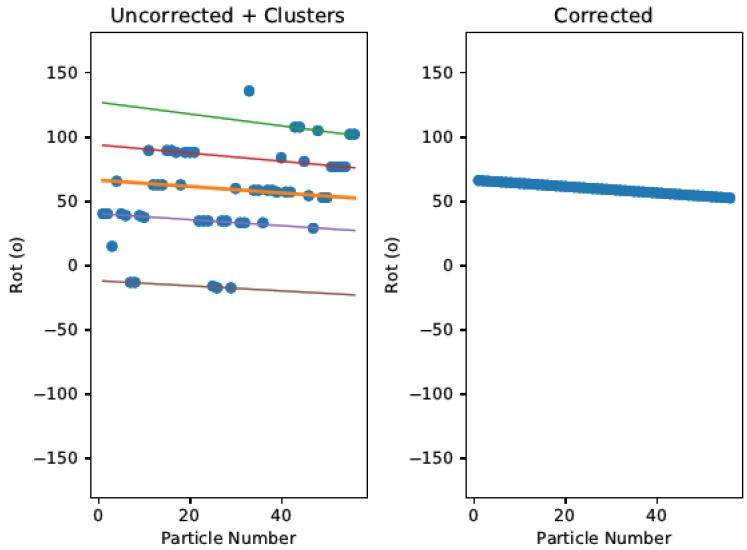

Figure 4: Distribution of Rot angles for an example microtubule. The plot on the left shows Rot angles plotted over microtubule particle number. The coloured lines show the clusters of Rot angles that MiRP has found. The bold line shows the modal cluster (cluster with the most particles). The plot on the right shows Rot angles after correction based on the modal cluster.

- Open /External/global_search/confidence.pdf. A confidence value (1-100%) for Rot angle assignment for each microtubule is calculated, and plotted as a histogram in this file. The more microtubules with higher confidence, the better Rot angle assignment has gone. However, Rot angle assignment is noisy, so you should expect a peak at 30-40 % confidence, for example in figure5.

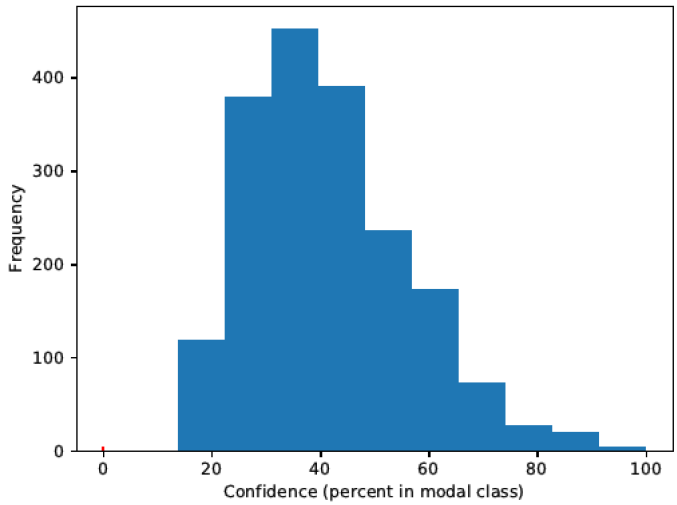

Figure 5: Example confidence plot for Rot angle assignment in the global search step. The confidence of Rot angle assignment for each microtubule is calculated (number of particles in modal cluster ÷total number of particles ×100), and plotted as a histogram here.

We now try to refine Rot angle assignment. Go to the 3D classification job to perform a local search. The parameters for this are the same as in the global search step, except for: Angular search range - rot, tilt, psi: 60, 15, 10.

After this job is complete, correct the Rot angles as above. You should see a slight shift towards higher confidence values in the confidence histogram.

3.3 X/Y shift assignment

The X/Y shifts centre microtubule particles on to the reference. For particles in a given microtubule, the X/Y shifts are related by the equation of a straight line. However, in the wide searches required to align particles to the reference, X/Y shifts may cause a particle to shift in tubulin register. This can cause duplicated data, or cause α/β-tubulin to shift out of register.

To perform an X/Y shift search, go to the Class3D job type:

- I/O:
  - Input: External/local_search/rotCorrected_data.star
  - Reference map: e.g. 14pf_kinesin-3HQD_5-56A.mrc
  - Reference mask: Use the same mask from the global search step.
- Reference:
  - Ref. map is on absolute greyscale? No.
  - Initial low-pass filter: 15.
  - Symmetry: C1.
- Optimisation
  - Number of classes: 1.
  - Number of iterations: 10.
  - Mask diameter: 90 % of box size.
- Sampling:
  - Angular sampling interval: 1.8.
  - Offset search range: approximately 84 Å
  - Offset search step: approximately 2 Å
- Helix:
  - Do helical reconstruction? Yes.
  - Tube diameter - inner, outer: 110, 390 (depending on presence/size of decorating protein.)
  - Angular search range - rot, tilt, psi: 12, 15, 10.
  - Range factor for local averaging: -1.
  - Keep tilt-prior fixed: Yes.
  - Apply helical symmetry? No.

Now perform X/Y shift correction using the External job type:

- Input
  - External executable: mirp_initial_seam
  - Input particles: input the run_it010_data.star file from your X/Y shift search job.
- Params
  - Param1 label, value: xy

You can now open the file External/your_job_name/XYcorrected.pdf. The graph on the left, shows the X and Y shifts plotted over particle number. You should expect that the shifts follow a clear straight line, but for some microtubules the line will 'jump' (see figure6 for an example). There will probably be several microtubules where the shifts are more variable, and some microtubules may be so variable that MiRP cannot figure out what the correct X/Y shifts should be. In this case, the alignment may not have worked well, or your data may not be of

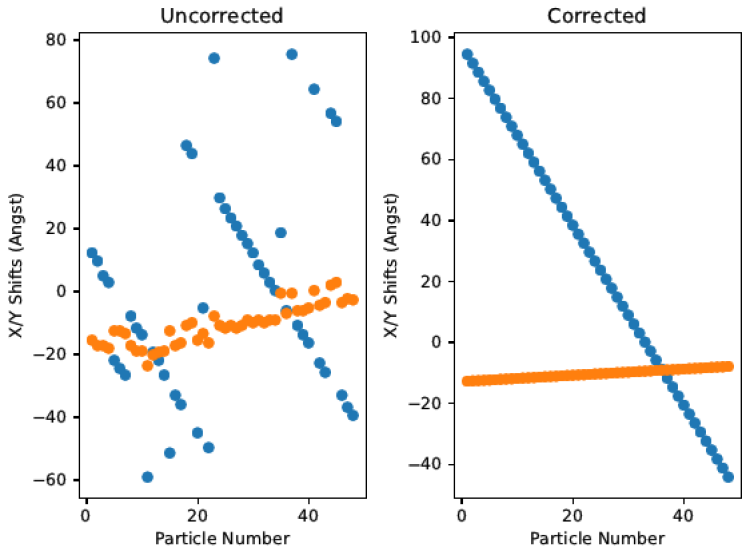

Figure 6: X/Y shift correction for an example microtubule. The plot on the left shows the uncorrected X/Y shifts (X = blue, Y = orange). For the X-shifts, there is a clear jump in value at particle 23. The change in value is around 80 Å (the approximate length of a αβ-tubulin dimer). The plot on the right shows the corrected X/Yshifts.

optimal quality.

We now need to revert to normal non-segment average particles before a final refinement. Use the following command:

```
generate_segment_averages -i ./External/mirp_pf_sorting/14pf_data.star -o ./External/mirp_xy_correct/ -r Extract/job_id/Micrographs
```

The -r flag specifies the path to the original particles, which will be used to update the starfile. A particles_reverted_data.star for use in RELION will be saved to the path specified with the -o flag.

Now perform a refinement with the original particles:

- I/O:
  - Input: External/xy_correct/XYcorrected.star
  - Reference map: e.g. 14pf_kinesin-3HQD_5-56A.mrc
  - Reference mask: Use the same mask from the global search step.
- Reference:
  - Ref. map is on absolute greyscale? No.
  - Initial low-pass filter: 15.
  - Symmetry: C1.
- Optimisation
  - Number of classes: 1.
  - Number of iterations: 10.
  - Mask diameter: 90 % of box size.
- Sampling:
  - Angular sampling interval: 1.8.
  - Offset search range: approximately 20 Å
  - Offset search step: approximately 2 Å

- Helix:
  - Do helical reconstruction? Yes.
  - Tube diameter - inner, outer: 110, 390 (depending on presence/size of decorating protein.)
  - Angular search range - rot, tilt, psi: 8, 10, 8.
  - Range factor for local averaging: -1.
  - Keep tilt-prior fixed: Yes.
  - Apply helical symmetry? No.

Check the output 3D reconstruction from this refinement. You should see the density for your decorating protein is more consistent between the different protofilaments, although still weaker at the seam.

4 Seam checking

Having achieved an initial seam alignment, we can now use supervised 3D classification to check the seam location for each microtubule.

4.1 Generating seam references

Seam checking requires a reference for each possible seam location, and for the two possible αβ-tubulin registers. For a 14 protofilament microtubule, this means 28 references are required. Seam references for kinesin and CAMSAP decorated microtubules are available in the data/seam_check. folder. If you want to generate seam references from your own reference, use this command, which like the generate_segment_averages script, is dependent on EMAN2:

```
generate_seam_references -i 14PF-kinesin.mrc -o seam_references -pf 14 -r 8.88 -p 1.39
```

where -i is the input 3D reference, -o is the folder to save the new references in, -pf is the protofilament number of the reference, -r is the rise in Å of the reference, and -p is the pixel size of your data.

4.2 Performing seam checking

To run the seam checking job, go to 3D classification and use the following settings:

- I/O:
  - Input: ./Refine3D/xy_refine/run_it010_data.star
  - Reference map: seam_refs.star
  - Reference mask: Do not input a mask for this job.
- Reference:
  - Ref. map is on absolute greyscale? No.
  - Initial low-pass filter: 15.
  - Symmetry: C1.
- Optimisation
  - Number of classes: 28 (for a 14 protofilament microtubule).
  - Number of iterations: 1.
  - Mask diameter: 90 % of box size.
- Sampling:
  - Perform image alignment?: No.
- Helix:
  - Do helical reconstruction? Yes.
  - Tube diameter - inner, outer: 110, 390 (depending on presence/size of decorating protein.)
  - Apply helical symmetry? No.

Now use MiRP to perform seam correction, using the External job type:

- Input
  - External executable: mirp_seam_check

  - Input particles: input the run_it001_data.star file from your seam classification job.

- Params
  - Param1 label, value: pf, 14 (replace with your protofilament number)
  - Param2 label, value: ri, 8.88 (replace with your helical rise)
  - Param3 label, value: c, 30

-c is the cutoff for the percent confidence in seam assignment for each microtubule, where any microtubule with a confidence below the cutoff is discarded. As per PF sorting, the confidence in seam class assignment for each microtubule (modal class ÷ total number of particles × 100) is calculated and plotted as a histogram in External/job_id/confidence.pdf (figure7). The right line shows the cutoff, and after seeing your distribution, you may wish to alter the cutoff to be more or less inclusive.

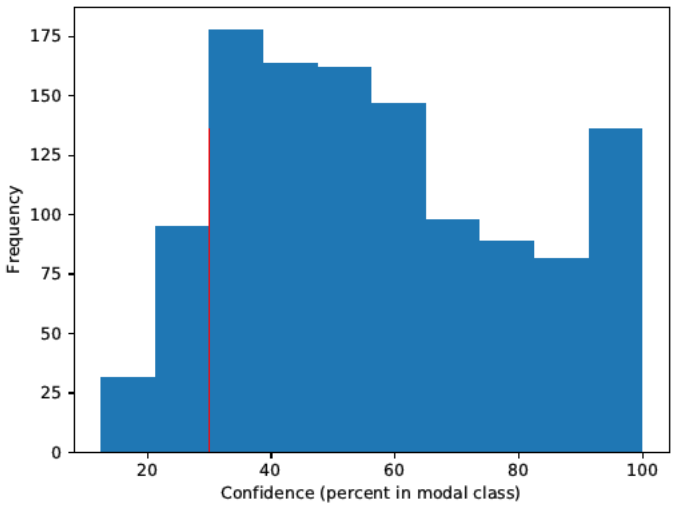

Figure 7: Histogram of the confidence of seam class assignment for each microtubule, from an example dataset.

MiRP also outputs External/job_id/seamclass_distribution.pdf, which tells you the percentage of your data that belongs to different seam classes. You should see that most microtubules belong to seam class 1, with for example, classes 2-7 gradually and classes 14-8 slowly decreasing in oc-

cupancy (e.g. figure8, which is for a kinesin). The percentage belonging to seam class1 depends on the size of decorating protein, how successful initial Rot angle assignment is, and the quality of your data. If this plot shows a very low percentage of data in seam class 1, or a seemingly random distribution in the different classes, then seam checking has not worked properly, or your data is of insufficient quality to proceed.

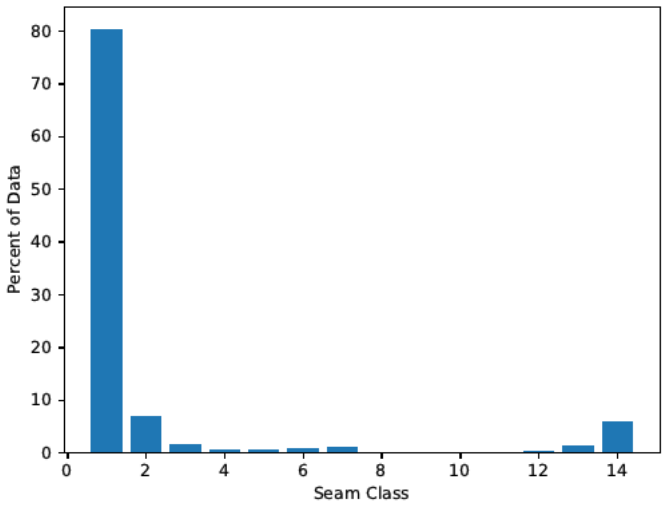

Figure 8: Percentage of data in different seam classes, as determined by the seam check step.

5 Tips/next steps

5.1 Assessing Euler angles and X/Y shifts

An additional useful diagnostic tool, is to plot the euler angles and X/Y shifts for each microtubule for visual inspection, which can be done with this command:

```
plot_eulerxy.py -i run_data.star -o run_data_plotted -n 50
```

where -i is the input starfile to plot microtubules from, -o is the output path and or file name for the plots - this is optional, and not including this option will bring up a display with the plots. -n (optional) is the number of microtubules to plot. This is useful with -o, as you probably don't need to look at every single microtubule, and this will speed up the plotting a lot. You can use this, for example, to check that most of your

microtubules are of good quality - fluctuating Psi and Tilt angles are a bad sign. You could use it to check that all your Rot and X/Y shifts are well aligned before you do the seam check step (e.g. figure 9)

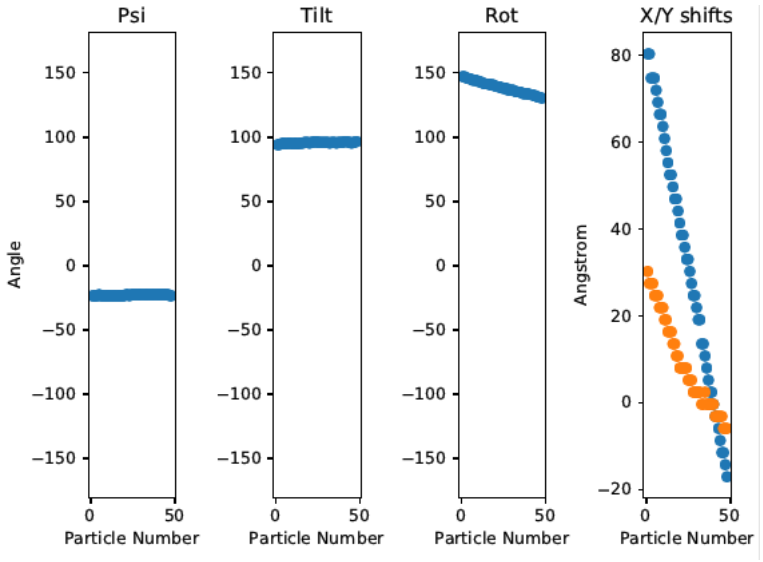

Figure 9: Euler angles and X/Y shifts plotted as a function of particle number, for an example microtubule before the seam checking step. This plot shows nice flat Psi and Tilt angles. In addition, all Rot angles follow a gentle slope (corresponding to the supertwist of the microtubule), and X/Y shifts follow a straight line.

5.2 C1 Reconstruction

If you are happy with the results of MiRP, you may now continue and use RELION for high resolution refinement and reconstruction of an asymmetric microtubule (C1 reconstruction). First, extract un-binned particles:

- I/O
  - OR re-extract refined particles? Yes.
  - Refine particles STAR file: External/job_id/seamCorrected_data.star
  - Reset the refined offsets to zero? No.
  - OR: re-center refined coordinates? No.
- extract

  - Particle box size: 600 Å (7.4 αβ-tubulin dimers), although smaller box sizes may be necessary to avoid memory constraints when using smaller pixel sizes.
  - Rescale particles? No.

- Helix
  - Extract helical segments? Yes.
  - Tube diameter: 390 Å (depending on size/presence of decorating protein).
  - Use bimodal angular priors? Yes.
  - Coordinates are start-end only? Yes.
  - Cut helical tubes into segments? Yes.
  - Number of unique asymmetrical units: 1.
  - Helical rise: 82.

You will require a 3D reference with box/pixel size corresponding to your un-binned particles. You will also need a binary soft edge mask, we have previously used the 3D reconstruction of class1 from seam checking for this, or a synthetic 3D reference, with a soft-edge of around 8 pixels. Use a central Z length of 30 %. Now go to the 3D-auto refine job type:

- I/O:
  - Input images STAR file: Extract/job_id/particles.star
  - Reference map: e.g. 14pf_kinesin-3HQD_1-39A.mrc
  - Reference mask: MaskCreate/job_id/mask.mrc
- Reference:
  - Ref. map is on absolute greyscale? No.
  - Initial low-pass filter: 15.
  - Symmetry: C1.

- Optimisation
  - Mask diameter (A): 75 % of box size.
  - Use solvent-flattened FSCs? Yes.
- Auto-sampling:
  - Initial angular sampling: 0.9 degrees.
  - Initial offset range (pix): approximately 20 Å.
  - Initial offset step (pix): approximately 2 Å.
  - Local searches from auto-sampling: 0.9 degrees.
  - Use finer angular sampling faster? No.
- Helix:
  - Do helical reconstruction? Yes.
  - Tube diameter - inner, outer: 110, 390 (depending on presence/size of decorating protein.)
  - Angular search range - rot, tilt, psi (deg): 8, 6, 10
  - Range factor for local averaging: -1.
  - Keep tilt-prior fixed: Yes.
  - Apply helical symmetry? No.

You should get a nice C1 reconstruction from this step. At this point you may want to try per-particle CTF refinement and/or bayesian polishing, which tend to improve resolution at least a little bit. There are also some other tips in the original MiRP publication that could be considered.

5.3 Symmetrised reconstruction

Once happy with the asymmetric reconstruction, you can now use symmetry to improve resolution (hopefully). There are three ways to do this.

1. Apply standard helical symmetry, as described in the original MiRP publication, to obtain a symmetrised asymmetric unit. For this option, perform a 3D auto-refine job using the same settings for a C1 reconstruction, except set 'Apply helical symmetry?' to 'Yes'. Then fill in the expected helical symmetry for your microtubule protofilament number (see DOI: http://dx.doi.org/10.1016/j.str.2010.05.010), and also perform local searches of symmetry. Because of the seam, application of helical symmetry introduces shifts in αβ-tubulin register, which destroys density corresponding to binding proteins, and differences between αβ-tubulin. Protofilaments next to the seam suffer the most, with the protofilament opposite the seam having no shifts in αβ-tubulin register introduced (symmetry is applied +/- 180°, rather than 360°). You can then extract density for an asymmetric unit opposite the seam for analysis/model building.

2. An alternative is to use RELION's local symmetry function to produce a symmetrised microtubule as very successfully done and described here: DOI - 10.7554/eLife.47145. You should incorporate the local symmetry method with the 3D auto-refine settings described here for C1 reconstruction.

3. Finally, you could use symmetry expansion, which will multiply and transform the particles by the number of protofilaments in the microtubule. To do this, use the RELION command line tool (this is an example for a 14 protofilament microtubule):

   ```
   relion_particle_symmetry_expand –i Refine3D/job_id/run_data.star –o Refine3D/job_id/run_data_expanded.star –sym C1 –helix –twist 27.5 –rise 1.39 –asu 14
   ```

   Each particle (each one theoretically corresponding to one unique protofilament) can then be refined independently in a 3D auto-refine run, using the same settings as described for C1 reconstruction. In practice, this often produces slightly higher resolution than the other symmetry application methods, although the refinements

obviously take a lot longer. The resulting map will be very similar to the one produced by applying standard helical symmetry - protofilaments nearer the seam will suffer from the introduction of particles with shifted αβ-tubulin register. As with standard helical symmetry, only the protofilament opposite the seam has no shifts in αβ-tubulin register introduced, and thus an asymmetric unit from this protofilament can be extracted and used for analysis.

A further option which was employed for *Plasmodium falciparum* kinesin-5, which showed poor microtubule decoration, is to use classification to select for decoration (DOI: https://doi.org/10.1101/2021.01.26.428220). To do this, one must make a mask for one kinesin binding site opposite the seam, in order to perform focussed 3D classification without alignment. Using for example three classes, one should obtain a single class which is mostly populated by kinesin, and one or two more with no kinesin.

6 Installation

MiRP should be easy to setup, however we have made an install.py script to try and do this automatically. In practice all this does is try to work out the current shell being used, and then add the path to MiRP to your environment. For example if install.py thinks you are using csh, it will add the following lines to the /.cshrc file:

```
setenv PATH ${PATH}:/d/pathtomirp//mirp/mirp
setenv PYTHONPATH /d/pathtomirp/mirp/mirp
```

7 Citing MiRP

In the first instance, please cite the original MiRP publication (DOI: 10.1016/j.jsb.2019.10.004). MiRP for use with RELION v3.1 is currently out in pre-print form: https://doi.org/10.1101/2021.01.26.428220.